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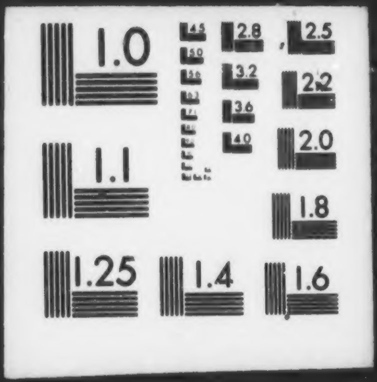
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# CHEMISTRY AND MODE OF ACTION OF INSECTICIDES

T. R. Fukuto

California University

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January 1976

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This report summarizes research accomplishments for the period January 1, 1971 to September 1, 1975. The study is concerned with the intoxication and detoxication processes which take place when an animal or plant is exposed to different organic insecticides. Progress in the following general areas is reported.

1. Insecticide selectivity.
2. Insecticide metabolism.
3. Inhibition of the cholinesterase enzymes.
4. Structure-activity relationships in insecticides.
5. Oxidative conversion of PS to PO esters.
6. Insecticide synergism and insect growth regulators.
7. Chemical reaction involving carbamate and organophosphorus esters.
8. Insecticide cyclic nucleotide interactions.
9. Insecticide penetration and its relation to resistance.
10. Gas chromatography of insecticides.
11. Neurophysiological studies on insecticide mode of action.
12. Insecticide resistance.
13. Joint action of insecticides and herbicides.

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# CHEMISTRY AND MODE OF ACTION OF INSECTICIDES

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## A. SUMMARY

This report summarizes research accomplishments for the period January 1, 1971, to September 1, 1975. In gross aspects, our studies on the chemistry and mode of action of insecticides have been concerned with the intoxication and detoxication processes which take place when an animal or plant is exposed to different organic insecticides. Progress in the following general areas is reported.

1. Insecticide selectivity
2. Insecticide metabolism
3. Inhibition of the cholinesterase enzymes
4. Structure-activity relationships in insecticides
5. Oxidative conversion of PS to PO esters
6. Insecticide synergism and insect growth regulators
7. Chemical reaction involving carbamate and organophosphorus esters
8. Insecticide cyclic nucleotide interactions
9. Insecticide penetration and its relation to resistance
10. Gas chromatography of insecticides
11. Neurophysiological studies on insecticide mode of action
12. Insecticide resistance
13. Joint action of herbicides and insecticides

## B. STUDIES ON SELECTIVE TOXICITY

### 1. Selective Toxicity of Phenylglyoxylonitrile Oxime O,O-Diethyl Phosphorothioate (Phoxim)

Introduction - Phoxim is a promising new organophosphorus insecticide which has shown excellent activity for the control of a variety of insects of hygienic and agricultural importance and is extremely safe to warm-blooded animals (rat oral $LD_{50}$ 8500 mg/kg). In contrast to most "safe" organophosphorus insecticides, Phoxim is unusual in that it is a diethyl ester rather than a dimethyl ester. Because of its outstanding properties of selectivity, an investigation on the mode of action of Phoxim in the white mouse and house fly was conducted to determine the basis for selectivity. In addition, a number of other dialkyl esters analogous to Phoxim were synthesized and examined for toxicity to mice and insects.

Metabolism in the White Mouse - $^{32}$P-Labeled Phoxim was given orally to white mice at 3 dosages; 10.5, 114 and 955 mg/kg. At these dosage levels, the ultimate recovery of administered radioactivity in the urine and feces ranged from 73 to 82% over a period of 140 hours. In general, excretion of radioactivity was substantially slower compared to other organophosphorus insecticides previously studie. For example, 24 hours after oral treatment of mice with 10.5 and 114 mg/kg of $^{32}$P-Phoxim only 43% and 22%, respectively of the administered radioactivity was excreted in the urine. At the high dosage of 955 mg/kg only 17% of the administered dosage was excreted after 30 hours.

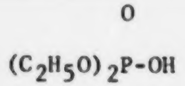
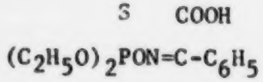
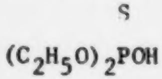
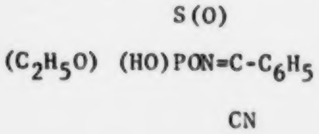
Because of the problem presented by the abnormally slow rate of Phoxim excretion, an autopsy was performed on a mouse treated at 114 mg/kg after 48 hours to determine the fate of the administered material. At this time 43% of the dose had been excreted. The results of the autopsy are presented in Table 1 and indicate that most of the radioactivity was localized in the gut or urinary bladder. Further, virtually all of the radioactivity was in the form of water soluble material, indicating that most of the administered dose was converted to detoxified materials. The slower than expected excretion rates observed after treatment with Phoxim is attributed to storage of metabolic products in the urinary bladder which was abnormally large owing to the accumulation of urine. An explanation for this observation is not available.

Table 2 gives data on the identity and relative amounts of the various metabolites obtained at 114 and 955 mg/kg. Radioactive urine from white mice was analyzed by ion-exchange and thin-layer chromatography (tlc). As indicated in the table, at least 5 metabolites were identified as (1) diethyl phosphoric acid, (2) Phoxim, (3) Phoxim carboxylic acid, (4) O,O-diethyl phosphorothioic acid, and (5) either desethyl Phoxim or desethyl PO Phoxim. Of the products isolated, diethyl

## Table 1. SUMMARY OF AUTOPSY DATA ON A WHITE MOUSE 48 HR AFTER ORAL TREATMENT WITH 114 MG/KG OF PHOXIM

| Organ | % Recovered internal radioactivity | | |
|---|---|---|---|
| | Organic-soluble | Water-soluble | Sum of organic- + water-soluble |
| Brain | 0.07 | 0.14 | 0.21 |
| Thymus gland | 0.01 | 0.05 | 0.06 |
| Hind leg muscle | 0.00 | 0.54 | 0.54 |
| Heart | 0.01 | 0.23 | 0.24 |
| Kidney | 0.03 | 0.13 | 0.16 |
| Liver | 0.05 | 1.60 | 1.65 |
| Gut | 0.17 | 8.60 | 8.77 |
| Urinary bladder | 2.10 | 86.30 | 88.40 |
| Total | 2.44 | 97.59 | 100.00 |

Table 2. METABOLITES FOUND IN URINE FROM WHITE MICE 24 HR AFTER TREATMENT WITH PHOXIM AT 114 MG/KG AND AFTER 30 HR AT 955 MG/KG

| | | Anion exchange tube numbers | 114 mg/kg dose | | 955 mg/kg dose | |
|---|---|---|---|---|---|---|
| | | | %[a] | mg[b] | % | mg |
| 1. |  $(C_2H_5O)_2\overset{O}{P}\text{-OH}$ | 21-35 | 58.9 | 0.62 | 43.1 | 2.36 |
| 2. | Phoxim | 47-57 | 1.1 | 0.01 | 2.1 | 0.12 |
| 3. |  $(C_2H_5O)_2\overset{S}{P}ON{=}\overset{COOH}{C}\text{-}C_6H_5$ | 47-57 | 2.8 | 0.03 | 23.6 | 1.29 |
| 4. |  $(C_2H_5O)_2\overset{S}{P}OH$ | 59-64 | 20.0 | 0.21 | 17.7 | 0.97 |
| 5. |  $(C_2H_5O)\ (HO)\overset{S(O)}{P}ON{=}\underset{CN}{C}\text{-}C_6H_5$ | 84-93 | 6.2 | 0.07 | 5.0 | 0.27 |
| | Radioactivity not in peaks | | 4.3 | 0.05 | 5.4 | 0.30 |
| | Unrecoverable radioactivity | | 6.7 | 0.07 | 3.1 | 0.17 |

---

[a] % of radioactivity in urine applied to column.
[b] Values expressed in terms of Phoxim equivalents based on average dose given to each mouse.

phosphoric acid and O,O-diethyl phosphorothioic acid were by far the most abundant metabolites.

Because of the large amounts of diethyl phosphoric acid present in the urine, $^{32}$P-O,O-diethyl phosphorothioic acid was given orally to the mouse to determine whether this material served as a precursor to diethyl phosphoric acid. However, this study showed that more than 95% of administered phosphorothioic acid was recovered in the urine as unchanged material suggesting that diethyl phosphoric acid was produced from PO Phoxim.

The most striking change in the relative amounts of any metabolite upon increasing the dose from 114 to 955 mg/kg occurred with Phoxim carboxylic acid. In terms of actual amounts of material present in the urine, Phoxim carboxylic acid was 43 times more abundant at the higher than at the lower dosage. The results point out the possible importance of this metabolic reaction in making Phoxim safe to mammals.

Metabolism in House Flies - The metabolism of Phoxim also was examined in susceptible ($S_{NAIDM}$) and resistant ($R_{SC}$) strains of house flies. $R_{SC}$ is the Stauffer chlorthion resistant strain to which Phoxim was about 100-fold less effective ($LD_{50}$ 215 μg/g) compared to the $S_{NAIDM}$ strain ($LD_{50}$ 2.1 μg/g). House flies were treated topically, $S_{NAIDM}$ at 1.75 μg/g and $R_{SC}$ at 1.75 and 150 μg/g. Penetration as measured by the rate of fly surface loss was rapid in both susceptible and resistant house flies, 80-90% being penetrated after 30 minutes. Penetration probably is not a factor contributing to the difference in Phoxim toxicity between the two strains.

The results showed conclusively that differences in the rates of detoxication of Phoxim exist between susceptible flies and the lower dosage and there are considerably larger amounts of hydrolytic products in the resistant strain at all times. Table 3 gives data on the nature and amounts of the metabolites found internally in flies 2 hours after treatment. The data show that five metabolites were found internally in the susceptible strain and six in the resistant strain. Phoxim, PO Phoxim, O,O-diethyl phosphorothioic acid, diethyl phosphoric acid, and phosphoric acid were found in both strains. A metabolite tentatively identified as ethyl phosphoric acid was found only in resistant flies. At the same dosage of 1.75 μg/g much smaller amounts of intact Phoxim and its PO analog were found in the internal extract of resistant compared to susceptible flies. The greatly reduced amounts of Phoxim and PO Phoxim in the resistant strain must be the result of more rapid metabolic degradation, resulting in significantly greater amounts of hydrolysis products in the resistant compared to the susceptible strain.

Toxicity of Phoxim Analogs - Toxicity data for Phoxim and some of its analogs against $S_{NAIDM}$ and $R_{SC}$ flies, German cockroach and the white mouse are summarized in Table 4. With the exception of the diethylphosphinothioate (IX) and phosphinate (X) analogs, all of the compounds were quite toxic to susceptible flies with $LD_{50}$ values between 2.1-16

Table 3. METABOLITES IN EXTRACT FRACTION FROM SUSCEPTIBLE AND RESISTANT HOUSE FLIES 2 HR AFTER TOPICAL APPLICATION OF PHOXIM

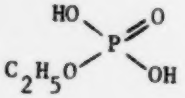
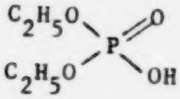
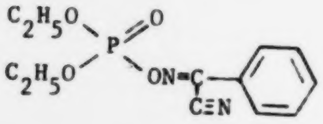
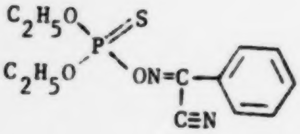
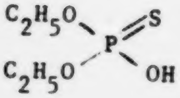
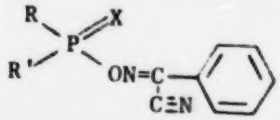
| Metabolite | | $S_{NAIDM}$ | $R_{SC}$ | |
|---|---|---|---|---|
| | | 1.75 µg/g Dose | 1.75 µg/g Dose | 150 µg/g Dose |
| $H_3PO_4$ | %<br>µg/g | 3.1<br>0.04 | 3.2<br>0.04 | 0.9<br>0.38 |
|  (?) | %<br>µg/g | -<br>- | 9.2<br>0.11 | -<br>- |
|  | %<br>µg/g | 26.9<br>0.38 | 43.3<br>0.53 | 11.9<br>5.1 |
|  | %<br>µg/g | 7.4<br>0.11 | 2.4<br>0.03 | 2.9<br>1.2 |
|  | %<br>µg/g | 30.3<br>0.39 | 5.9<br>0.38 | 47.9<br>14.7 |
|  | %<br>µg/g | 27.6<br>0.39 | 30.6<br>0.38 | 34.4<br>14.7 |

Table 4. SUMMARY OF TOXICITY DATA FOR PHOXIM AND ITS ANALOGS TO HOUSE FLIES ($S_{NAIDM}$ AND $R_{SC}$), GERMAN COCKROACHES, AND WHITE MICE



| Analog | R | R' | X | LD$_{50}$ Values | | | |
|---|---|---|---|---|---|---|---|
| | | | | Insects (µg/g) | | | |
| | | | | $S_{NAIDM}$ flies | $R_{SC}$ flies | German cockroach | White mice (mg/kg) |
| I | $C_2H_5O$ | $C_2H_5O$ | S | 2.1 | 215 | 6.1 | >2,000 |
| II | $C_2H_5O$ | $C_2H_5O$ | O | 3.4 | >2,500 | 5.5 | 1,000 |
| III | $CH_3O$ | $CH_3O$ | S | 3.3 | >2,500 | 7.8 | >2,000 |
| IV | $CH_3O$ | $CH_3O$ | O | 5.0 | >2,500 | 7.6 | >1,000 |
| V | $i\text{-}C_3H_7O$ | $i\text{-}C_3H_7O$ | S | 16.0 | 220 | 209 | >1,500 |
| VI | $i\text{-}C_3H_7O$ | $i\text{-}C_3H_7O$ | O | 5.0 | 53.5 | 12.5 | 1,250 |
| VII | $C_2H_5O$ | $C_2H_5$ | S | 5.8 | 21.5 | 9.7 | >500 |
| VIII | $C_2H_5O$ | $C_2H_5$ | O | 5.9 | 150 | 5.5 | 70-73 |
| IX | $C_2H_5$ | $C_2H_5$ | S | 125 | >2,500 | - | >500 |
| X | $C_2H_5$ | $C_2H_5$ | O | 410 | >2,500 | - | >500 |

µg/g. Toxicities against resistant flies were variable, ranging from 21.5 to >2,500 µg/g, and with the exception of V, the analogs showed high toxicity to the German cockroach. However, with the exception of the ethylphosphonate analog, all compounds were of low toxicity to the white mouse.

The data in Table 5 show that there are substantial differences in the sensitivity of insect and mammalian ChE to inhibition by the various PO esters. The magnitude of these differences is represented in Table 5 by the ratios of inhibition of the two cholinesterases, i.e., $k_e$ (flyhead ChE)/$k_e$ (bovine erythrocyte ChE) defined as the selective inhibition ratio. The largest ratio was found with X, the diethylphosphinate analog of Phoxim. However, in spite of the large difference in inhibition rates, X was nontoxic to both the house fly and the mouse probably because of its high susceptibility to hydrolysis. Compounds II, IV, and VI all showed large selective inhibition ratios and all these compounds were substantially more toxic to house flies than to mice. The phosphonate analog VIII, the compound most toxic to the mouse, showed the lowest inhibition ratio of 49.

Conclusion - Our results indicate the metabolic factors play an important role in the biocidal activity of Phoxim to insects and the mouse. The equations below show the proposed scheme for the metabolism of Phoxim in the white mouse and in house flies.

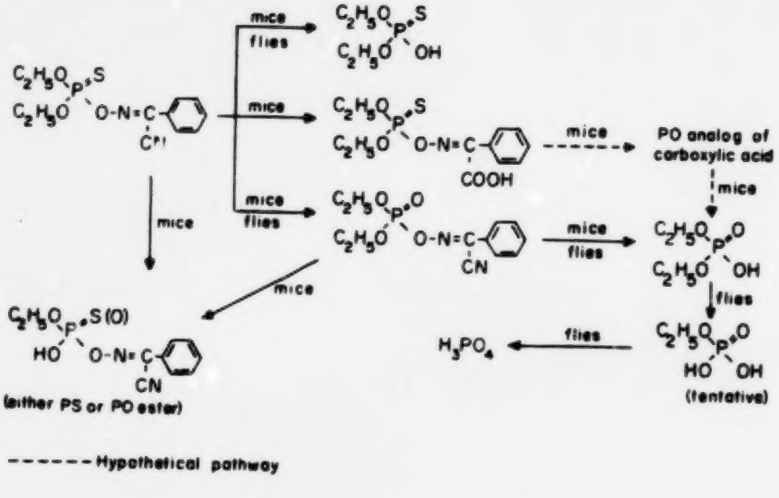
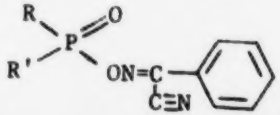

mice
flies
mice
mice
flies
mice
mice
flies
mice
mice
flies
flies
PO analog of carboxylic acid
COOH
CN
H$_3$PO$_4$
(either PS or PO ester)
(tentative)
------Hypothetical pathway

Table 5. CHOLINESTERASE INHIBITION DATA FOR PHOXIM AND ITS ANALOGS AT 37.5°



| Analog | R | R' | SNAIDM Fly-head ChE |  | Bovine-erythrocyte ChE |  | $k_e$ (fly)/$k_e$ (bovine) | $k_b$ ($min^{-1}$) 30° |
|---|---|---|---|---|---|---|---|---|
|  |  |  | $I_{50}$ (M) | $k_e$ ($M^{-1}$, $min^{-1}$) | $I_{50}$ (M) | $k_e$ ($M^{-1}$, $min^{-1}$) |  |  |
| I | $C_2H_5O$ | $C_2H_5O$ | $1.9 \times 10^{-9}$ | $8.4 \times 10^{7}$ | $2.2 \times 10^{-7}$ | $3.1 \times 10^{5}$ | 270 | $5.9 \times 10^{-2}$ |
| IV | $CH_3O$ | $CH_3O$ | $1.1 \times 10^{-9}$ | $1.3 \times 10^{8}$ | $3.6 \times 10^{-7}$ | $1.7 \times 10^{5}$ | 730 | $1.4 \times 10^{-1}$ |
| VI | $i-C_3H_7O$ | $i-C_3H_7O$ | $7.8 \times 10^{-10}$ | $8.1 \times 10^{7}$ | $4.4 \times 10^{-7}$ | $1.1 \times 10^{5}$ | 756 | $2.7 \times 10^{-2}$ |
| VIII | $C_2H_5O$ | $C_2H_5$ | $2.5 \times 10^{-9}$ | $5.9 \times 10^{7}$ | $5.1 \times 10^{-8}$ | $1.2 \times 10^{6}$ | 49 | $3.9 \times 10^{-1}$ |
| X | $C_2H_5$ | $C_2H_6$ | $1.2 \times 10^{-8}$ | $1.9 \times 10^{6}$ | $3.8 \times 10^{-5}$ | $8.3 \times 10^{2}$ | 2,330 | $7.1 \times 10^{-1}$ |

The basis for the extreme safety of Phoxim to mice is readily apparent from the metabolism data. Only very small amounts of Phoxim were found in mouse urine 24 hr after treatment and in no case was any PO Phoxim detected. Further, only insignificant amounts of intact ester were noted in the internal organs of the mouse autopsied 48 hr after treatment with 114 mg/kg Phoxim. The evidence clearly indicates that Phoxim is peculiarly suited for metabolic detoxication by the white mouse. Owing to the large amounts of diethyl phosphoric acid found in mouse urine, it is evident that Phoxim is desulfurated to PO Phoxim which in turn must be hydrolyzed with remarkable rapidity to diethyl phosphoric acid and at no time does PO Phoxim reach a sufficiently high concentration to intoxicate the mouse. Support for rapid degradation of PO Phoxim is found in the fact that PO Phoxim itself is quite nontoxic with an $LD_{50}$ value of 1000 mg/kg. In addition to the above pathway for detoxication, the mouse also is capable of degrading Phoxim directly to *O*,*O*-diethyl phosphorothioic acid and to Phoxim carboxylic acid. The hydrolysis of Phoxim to *O*,*O*-diethyl phosphorothioic acid appears to be relatively constant with respect to the administered dose. Hydrolysis to Phoxim carboxylic acid, on the other hand, appears to increase with increase in administered dosage, and evidently becomes an important detoxication pathway at high dosages. The net result is that PO Phoxim in spite of its strong anticholinesterase properties, is not allowed to reach a level critical in the mouse.

## 2. Metabolism of Abate in Mosquito Larvae and House Flies.

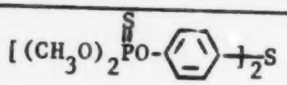
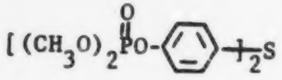
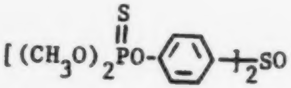
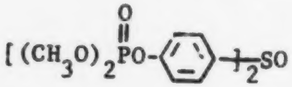
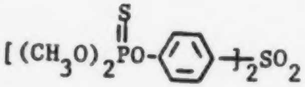
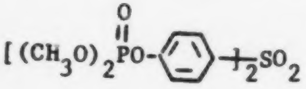
*Introduction* - Abate (*O*,*O*,*O*',*O*'-tetramethyl *O*,*O*'-thiodi-*p*-phenylene phosphorothioate) is currently being used for the control of aquative larvae. Based on extensive toxicological evaluations, Abate has proved to be highly effective against a variety of mosquito larvae but is relatively nontoxic to other insects, fish, and mammals. The reported $LD_{50}$ of Abate to rats and mice is approximately 4000 mg/kg. Abate, because of its peculiar specificity toward aquatic larvae, provides an interesting example of selectivity between insects. The following discussion is concerned with the absorption and metabolism of Abate in the larvae of the mosquito (*Aedes aegypti*) and common house fly.

*Toxicity of Abate and Some of its Oxidation Products* - The principal oxidation products of Abate were synthesized and examined for toxicity to mosquito larvae and house flies. The data in Table 6 show that these compounds are relatively nontoxic to the house fly. Against the 4th instar mosquito larvae, *Aedes aegypti*, Abate also was more effective than its oxidation products and the order of effectiveness may be summarized as follows:

$$PS(S) > PS(SO) > PS(SO_2) > PO(S) > PO(SO) > PO(SO_2).$$

Evidently, the order of toxicity to mosquito larvae is directly related to the oxidation state of the molecule. Although this order may be purely fortuitous, i.e., decreasing toxicity with increasing oxidation

Table 6. THE CHEMICAL AND TOXICOLOGICAL PROPERTIES OF ABATE AND ITS ANALOGS

| Compound structure | | Designated symbol | MP (°C) | Elemental analysis | | Insect toxicity | |
|---|---|---|---|---|---|---|---|
| | | | | Calcd | Found | House fly $LD_{50}$ (µg/g) | Mosquito larvae $LC_{50}$ (ppm) |
| XI |  | PS(S) | 36 | - | - | 205 | 6.3 X $10^{-3}$ |
| XII |  | PO(S) | 1.5522[a] | C 44.22<br>H 4.60 | 44.50<br>5.56 | >500 | 6.3 X $10^{-2}$ |
| XIII |  | PS(SO) | 41-42 | C 39.65<br>H 4.14 | 39.53<br>4.18 | >500 | 3.3 X $10^{-2}$ |
| XIV |  | PO(SO) | 32-33 | C 42.60<br>H 4.44 | 42.46<br>4.24 | >500 | 2.4 X $10^{-1}$ |
| XV |  | PS($SO_2$) | 61-62 | C 38.52<br>H 4.03 | 38.38<br>4.07 | >500 | 3.9 X $10^{-2}$ |
| XVI |  | PO($SO_2$) | 85-87 | C 41.15 | 41.36 | >500 | 2.9 X $10^{-1}$ |

[a] Liquid, refractive index.

state (and also polarity), it nevertheless suggests that absorption of the toxicant into the larvae from the aqueous phase may strongly influence the effectiveness of the compound. PS esters, being more lipophilic than PO esters, should be able to penetrate the hydrophobic epicuticular wax layer of the larvae at a faster rate compared to PO esters. The same reasoning also applies to the relative toxicities of the sulfide, sulfoxide, and sulfone.

No obvious relationship was evident between toxicity and anticholinesterase activity of the PO esters (XIV-XVI) and it appears that sensitivity of fly-head and mosquito larvae cholinesterase toward these has little to do with the selective properties of Abate. The pattern of anticholinesterase activity against mosquito larvae cholinesterase was in the order expected, i.e., $PO(SO_2) > PO(SO) > PO(S)$. Overall, however, the series of PO esters were equally effective in inhibiting fly-head and mosquito larvae cholinesterase, suggesting that cholinesterase inhibition has little to do with selectivity.

Metabolism in the House Fly - House flies were treated topically with $^3H$-Abate at the dosages of 4.23 and 53.8 μg/g. Recovery of total radioactivity was quite good at the higher dosage (84-100%) but was less satisfactory at the lower dosage which and an average recovery of only 60%. The fraction of applied Abate penetrated in the house fly was much greater at the lower dosage and was approximately equal to that of other organophosphorus insecticides previously studied, e.g., Phoxim. At the higher dosage, the amount of penetrated Abate relative to the applied dose was low and the rate of penetration was approximately zero-order, indicating that the penetration process was near saturation at this dosage.

Virtually all of the expected metabolic products were found internally in the fly, either as the intact ester, or as hydrolyzed material. Table 8 gives the various amounts, in terms of microgram equivalents of Abate per gram of fly for each metabolite found in the internal fraction after treatment with Abate. The data show that there is a significant lag in the accumulation of total intact esters (XI-XVI) at the higher dosage relative to the amount of applied Abate. For example, at the point where approximately maximum accumulation occurs (5 hr) and where comparison can be made, the total amount of intact esters at the high dosage was 5.88 μg/g compared to 0.85 μg/g at the low dosage. Thus, the ratio of intact ester present internally at the two dosages is 6.8, a significantly smaller number than the 12.3-fold difference in the applied dosages. At the earlier time periods, the ratio was even smaller. These results serve to point out the limiting effect that penetration has on house fly intoxication by Abate.

The largest amount of radioactivity present in the fly was in the form of unchanged Abate at all time intervals after treatment, ranging from 75-90% of the total intact esters, indicating that the metabolism of

Table 7. BIMOLECULAR RATE CONSTANTS ($k_i$) FOR THE INHIBITION OF HOUSE FLY-HEAD AND MOSQUITO LARVAE CHOLINESTERASE BY PO ANALOGS OF ABATE

| Compound | | $k_i$ ($M^{-1}$ $min^{-1}$) X $10^{-6}$ | |
|---|---|---|---|
| | | House fly | Mosquito larvae |
| XIV | PO(S) | 6.99 | 0.37 |
| XV | PO(SO) | 0.59 | 1.10 |
| XVI | PO($SO_2$) | 1.20 | 1.56 |

Table 8. AMOUNTS OF ABATE AND ITS METABOLITES FOUND INTERNALLY IN THE FLY AFTER TREATMENT WITH ABATE

| Compound | Abate dosage (μg/g) | Amount (μg Abate equiv/g) of recovered radioactivity at indicated time intervals (hr) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.25 | 0.5 | 1.0 | 2.0 | 4.0 | 5.0 | 6.0 | 7.0 | 10.0 |
| XI PS(S) (Abate) | 53.8 | 0.62 | 0.98 | 1.37 | 1.90 | 4.25 | 4.67 | 5.16 | 4.55 | - |
| | 4.23 | 0.22 | - | - | 0.50 | - | 0.68 | - | - | 0.46 |
| XII PS(SO) | 53.8 | 0.04 | 0.04 | 0.07 | 0.13 | 0.46 | 0.61 | 0.70 | 0.85 | - |
| | 4.23 | 0.01 | - | - | 0.04 | - | 0.08 | - | - | 0.04 |
| XIII PS($SO_2$) | 53.8 | 0.02 | Tr[a] | ND[b] | 0.02 | 0.10 | 0.20 | 0.19 | 0.21 | - |
| | 4.23 | ND | Tr | - | 0.01 | - | 0.04 | - | - | 0.02 |
| XIV PO(S) | 53.8 | ND | Tr | 0.01 | 0.04 | 0.11 | 0.14 | 0.13 | 0.12 | - |
| | 4.23 | Tr | - | - | 0.01 | - | 0.02 | - | - | 0.01 |
| XV PO(SO) | 53.8 | ND | ND | 0.01 | 0.04 | 0.12 | 0.14 | 0.22 | 0.18 | - |
| | 4.23 | Tr | - | - | 0.01 | - | 0.02 | - | - | 0.02 |
| XVI PO($SO_2$) | 53.8 | ND | ND | Tr | 0.02 | 0.09 | 0.12 | 0.10 | 0.11 | - |
| | 4.23 | Tr | - | - | Tr | - | 0.01 | - | - | 0.01 |
| XVII HO(S) | 53.8 | ND | ND | ND | ND | ND | Tr | Tr | Tr | - |
| | 4.23 | Tr | - | - | Tr | - | Tr | - | - | Tr |
| XVIII HO(SO) | 53.8 | ND | 0.01 | 0.04 | 0.19 | 0.18 | 0.24 | 0.32 | 0.14 | - |
| | 4.23 | 0.01 | - | - | 0.02 | - | 0.03 | - | - | 0.02 |
| XIX HO($SO_2$) | 53.8 | ND | ND | ND | ND | ND | Tr | 0.02 | 0.02 | - |
| | 4.23 | Tr | - | - | Tr | - | 0.01 | - | - | 0.01 |
| Conjugates ($R_fO$) | 53.8 | 0.09 | 0.11 | 0.33 | 0.51 | 1.10 | 1.14 | 1.83 | 1.34 | - |
| | 4.23 | 0.02 | - | - | 0.19 | - | 0.11 | - | - | 0.01 |

Table 8 (continued).

| Compound | Abate dosage (μg/g) | Amount (μg Abate equiv/g) of recovered radioactivity at indicated time intervals (hr | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.25 | 0.5 | 1.0 | 2.0 | 4.0 | 5.0 | 6.0 | 7.0 | 10.0 |
| Total PS esters | 53.8 | 0.68 | 1.02 | 1.44 | 2.05 | 4.81 | 5.48 | 6.05 | 5.62 | - |
| | 4.23 | 0.23 | - | - | 0.55 | - | 0.80 | - | - | 0.52 |
| Total PO esters | 53.8 | ND | Tr | 0.02 | 0.10 | 0.32 | 0.40 | 0.45 | 0.41 | - |
| | 4.23 | Tr | - | - | 0.02 | - | 0.05 | - | - | 0.04 |

---

[a] Trace.

[b] Not detectable.

Abate to oxidation products is quite slow in the house fly. Of the various oxidative metabolites, Abate sulfoxide (XII) was present in substantially larger amounts than any of the other intact esters. Significant amounts of the PO esters (XIV-XVI) were not found until 1 hr after treatment and the amount reached a maximum at around 5-6 hr. The total amount of PO esters accumulating in the house fly was much lower than that found for PO esters generated from other phosphorothionates previously studied in this laboratory. Since intoxication of house flies probably occurs through the PO esters, i.e., by inhibition of cholinesterase, the low level of PO esters present after Abate treatment is consistent with the high tolerance of house flies toward Abate.

Metabolism in Mosquito Larvae - A single dose of 0.0565 ppm Abate in 3.0 ml water containing 0.25 g larvae was used for the metabolism study in mosquito larvae. Because of its aqueous habitat, it was necessary to examine the water medium for metabolites as well as the larvae. It became evident early in our studies that the larvae absorbed Abate at a rapid rate and that the metabolites appear both internally in the larvae and externally in the water medium after short intervals following exposure. This absorption and desorption phenomenon is termed "recycling." The absorption of Abate into mosquito larvae was extremely rapid and over 99% was absorbed within 1 hour after exposure. In determining the rate of absorption, the amount of Abate remaining in the water was assumed to be unabsorbed material.

In spite of the recycling phenomenon, substantial amounts of radioactivity remained inside the larvae. Data for the distribution of Abate and its metabolites in larvae and the water medium are summarized in Table 9. The amount present in water was divided into an ether-extractable and an ether-unextractable fraction to differentiate between metabolites and conjugates since compounds XI-XIX all partitioned in favor of ether. The total recovery of radioactivity, including that found in the larvae, ether-extractables and ether-unextractables in the water medium, ranged from 80-91%. Examination of the data will show that substantial amounts of radioactivity accumulate in the larvae, even as early as 1/4 hr after exposure, rising to a maximum after 1 hr (55% of recovered dosage). Evidently, Abate or its metabolites accumulate in mosquito larvae at a rate some 2-3 times faster than in house flies.

The data in Table 9 show that the largest amount of radioactivity found internally in mosquito larvae was in the form of Abate. However, in contrast to house flies, Abate appeared to be more rapidly metabolized in mosquito larvae. This is apparent from the amounts of phenols (XVII-XIX) and conjugates listed in the table. The amount of PO esters found at any time after treatment is the result of the difference in formation and degradation of these esters. Since mosquito larvae are able to degrade Abate and its intact metabolites to hydrolytic products faster than house flies, it appears that the conversion of PS to PO esters takes

Table 9. AMOUNTS OF ABATE AND ITS METABOLITES IN MOSQUITO LARVAE AND WATER MEDIUM (ETHER EXTRACT) AFTER EXPOSURE TO 0.687 μG/G ABATE

| Compound | Amount (μg Abate equiv/g) of recovered radioactivity at indicated time intervals (hr) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.25 | 0.5 | | 1.0 | | 2.0 | | 4.0 | | 5.0 | |
| | Larvae | Larvae | Water | Larvae | Water | Larvae | Water | Larvae | Water | Larvae | Water |
| XI Abate | 0.183 | 0.211 | 0.065 | 0.120 | 0.009 | 0.071 | 0.011 | 0.049 | 0.004 | 0.053 | 0.002 |
| XII PS(SO) | 0.004 | 0.002 | 0.111 | 0.003 | 0.059 | 0.004 | 0.026 | 0.004 | 0.016 | 0.003 | 0.011 |
| XIII PS($SO_2$) | 0.002 | 0.001 | 0.002 | 0.009 | 0.010 | 0.008 | 0.002 | 0.001 | 0.004 | Tr[a] | 0.014 |
| XIV PO(S) | 0.003 | 0.001 | 0.002 | 0.001 | 0.010 | 0.001 | 0.003 | 0.001 | 0.007 | Tr | 0.003 |
| XV PO(SO) | 0.005 | 0.002 | 0.001 | 0.026 | 0.004 | 0.005 | 0.003 | 0.001 | 0.005 | 0.001 | 0.001 |
| XVI PO($SO_2$) | 0.001 | 0.001 | 0.001 | 0.005 | 0.002 | 0.002 | 0.001 | 0.001 | 0.005 | 0.001 | 0.003 |
| XVII HO(S) | Tr | 0.001 | Tr | ND[b] | ND | 0.001 | ND | ND | ND | Tr | Tr |
| XVIII HO(SO) | 0.023 | 0.019 | 0.001 | 0.022 | 0.005 | 0.048 | 0.009 | 0.021 | 0.012 | 0.033 | 0.002 |
| XIX HO($SO_2$) | 0.003 | 0.003 | 0.002 | Tr | 0.002 | ND | 0.005 | 0.003 | 0.017 | 0.007 | 0.017 |
| Conjugates ($R_f0$) | 0.061 | 0.059 | 0.003 | 0.184 | 0.010 | 0.205 | 0.013 | 0.170 | 0.016 | 0.144 | 0.016 |
| Total PS esters | 0.189 | 0.214 | 0.178 | 0.132 | 0.078 | 0.083 | 0.039 | 0.055 | 0.024 | 0.056 | 0.027 |
| Total PO esters | 0.009 | 0.004 | 0.004 | 0.032 | 0.016 | 0.008 | 0.007 | 0.003 | 0.017 | 0.002 | 0.007 |

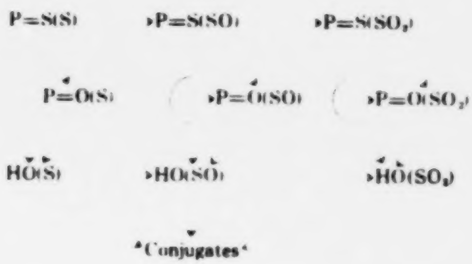
[a] Trace.

[b] Not detectable.

place more rapidly in mosquito larvae than in house flies. For example, at 1 hr after treatment, approximately 7.1% (0.048 μg/g) of the applied dose of 0.678 μg/g was present as PO esters (XIV-XVI) in the total system (larvae and water medium). In comparison, house flies treated at 4.23 μg/g, a dosage some 16-fold greater than for mosquito larvae on a weight basis, contained 0.02 μg/g (0.59% of applied dosage) at 2 hr following treatment.

The data in Table 9 show that significant amounts of intact esters are present in the water at all time periods, the largest fraction consisting of the PS(SO) derivative (XII). These results indicate that Abate, after absorption in the larvae, is rapidly converted to metabolic products and a substantial portion of the metabolites is eliminated into the water medium, including intact esters. These materials, then, are available to the mosquito larvae for reabsorption and further metabolism.

Conclusion - The pathways for the metabolism of Abate in the house fly and mosquito larvae are qualitatively similar as indicated in the scheme below. No gross difference in metabolic pathways or products was noted which would readily explain the selective action of Abate.



In spite of the overall similarity in the behavior of Abate in house flies and mosquito larvae, there are distinct quantitative differences which may account, at least in part, for the toxicity of Abate to these insects. A notable difference is the apparently greater rate of Abate absorption into mosquito larvae compared to flies. At the dosage of 0.678 μg/g (0.3 ml of 0.0565 ppm Abate containing 0.25 g larvae), the penetration of Abate into mosquito larvae was extremely rapid and virtually all of the Abate in the water medium was absorbed into the larvae in less than 1 hr after exposure. The dosage of 0.678 μg/g is approx 27-fold less than the calculated $LC_{50}$ value of 18 μg/g Abate to larvae (from an $LC_{50}$ value of 0.0063 ppm Abate in 100 ml water containing 20 larvae.

In terms of actual amounts penetrated, house flies absorbed only 2.6 times as much Abate in 1 hr compared to mosquito larvae, although the dosage applied to flies (4.23 μg/g) was more than 6-fold greater than that applied to mosquito larvae. The relative rate of penetration in house flies after treatment at 53.8 μg/g, a dosage approx 4-fold less than the $LD_{50}$ value, was much slower and may be the limiting step in the intoxication process.

The accumulation of intact PO esters produced by desulfuration of PS esters also was greater in mosquito larvae compared to house flies, particularly in the early stages after treatment where as much as 8-fold more PO ester was present in the larvae. The rapid buildup of PO esters in the mosquito larvae may be attributed in part to rapid initial absorption and the recycling process which allows reabsorption of previously eliminated intact esters from the water medium. The recycling phenomenon particularly applies to the PS(SO) ester which was present in relatively large amounts in the water medium shortly after larvae exposure. Rapid absorption of Abate and conversion to intact PO esters in the larvae undoubtedly are important factors which contribute to the peculiar specificity of Abate to mosquito larvae.

### 3. Metabolism of 2,2-Dimethyl-2,3-Dihydrobenzofuranyl-7 N-Dimethoxyphosphinothioyl-N-Methylcarbamate (PSC) in the House Fly, Rat and Mouse

Introduction - A previous investigation in this laboratory demonstrated that toxic methylcarbamate insecticides may be converted into derivatives possessing favorable properties of selectivity by appropriate substitution of the proton on the carbamyl nitrogen atom[1]. Compared to the original methylcarbamate, the N-dialkoxyphosphinothioyl moieties are substantially less toxic to the white mouse but on the whole are quite effective insecticides. In seeking additional information concerning the underlying basis for the selective toxicity of derivatized methylcarbamate esters, a study of the comparative metabolism of the dimethoxyphosphinothioyl derivative of carbofuran (XXX, 2,2-dimethyl-2,3-dihydrobenzofuranyl-7 N-dimethoxyphosphinothioyl-N-methylcarbamate, or PSC) was conducted in the house fly, mouse, and rat.

Metabolism in the White Mouse and Rat - White mice and rats were treated orally at a dose of 100 mg/kg of $^{14}C$ ring-labeled PSC. At this dosage, PSC was metabolized rapidly and excreted principally *via* the urine. For example, in the mouse 64% of the administered dose was recovered after 21 hours, 78% after 24 hours, and 88% after 96 hours. A maximum of 5% of the dose was recovered in the feces during a 96-hour collection period. The total recovery of $^{14}C$ ranged from 93-95%.

Of the total radioactivity present in the 24-hr urine sample, less than 1% was extractable into chloroform and the bulk of the radioactivity remained in the aqueous phase, presumably in the form of conjugates. Mild acid hydrolysis of the aqueous phase, however, afforded products in virtually quantitative conversion which were extractable into chloroform.

Analysis of the chloroform extract by tlc revealed the presence of the seven metabolites listed in Table 10, of which two remain as minor unknown components. 3-Ketocarbofuran and 3-hydroxycarbofuran were identified by cochromatography with authentic samples and cochromatography, infrared and mass spectral analyses were used to verify the structures of carbofuran phenol, 3-hydroxycarbofuran phenol and 3-ketocarbofuran phenol.

The data in Table 10 reveal that a major fraction of the administered $^{14}$C-ring PSC is eliminated in the urine as conjugated hydrolytic products. For example, approximately 72% of the radioactivity recovered from the mouse was isolated as the phenols of carbofuran, 3-hydroxycarbofuran, and 3-ketocarbofuran, and these materials represented 76% of the radioactivity recovered from the rat. Although significant amounts of 3-hydroxycarbofuran and 3-ketocarbofuran also were detected, these metabolites appeared after mild acid hydrolysis and evidently were present in the urine as conjugates. 3-Hydroxycarbofuran is somewhat toxic to the white mouse ($LD_{50}$ 7 mg/kg) and conjugation evidently is necessary to avoid or reduce intoxication by this metabolite.

$^{32}$P-PSC also was administered to mice at 100 mg/kg in order to establish the nature of the fragmentation products associated with the dimethoxyphosphinothioyl portion of the molecule. After treatment, radioactivity was excreted in the urine with up to 70% of the dose recovered in 24 hr. This was followed by a slower excretion rate, resulting in 93% total recovery in 72 hr. In contrast to the $^{14}$C ring-labeled metabolites, 55-75% of the radioactivity in the 24-hr urine sample was chloroform extractable, the rest remained in the aqueous phase even after adjustment of acidity to pH 1. Throughout the remainder of the observation period, the fraction of chloroform extractable radioactivity decreased to 20% for the 48-hr sample (24-48 hr) and 12% for the 72-hr sample (48-72 hr). A similar metabolic behavior was observed in the rat and pooled chloroform extracts were used for isolation of the major metabolite.

A maximum of 40% of the dose was present in the feces, most of which was unchanged PSC along with minor amounts of unidentified polar metabolites.

The pooled chloroform-soluble extract was analyzed by silica gel tlc using ethanol-acetone (9:1) for development. After repeated tlc and extraction of the $R_f$ 0.71 zone with chloroform, a single metabolite was obtained which was further purified by preparative glc. Subsequent analysis by glc-ms revealed a parent ion of 139 which, along with its fragmentation products, verified the structure of the metabolite as dimethyl N-methylphosphoramide (XXXVIII). A similar correspondence was obtained between the metabolite and an authentic sample of dimethyl N-methylphosphoramide by infrared and tlc analysis.

Preliminary ion exchange chromatography revealed that at least four additional $^{32}$P metabolites were present in the aqueous phase, each in minor amounts, but none were identified.

Table 10. METABOLITES RECOVERED FROM WHITE MOUSE AND RAT EXCRETA 24 HOURS AFTER ORAL ADMINISTRATION OF 100 MG/KG $^{14}$C-RING LABELED DIMETHOXYPHOSPHINOTHIOYL CARBOFURAN

| | | Per cent[a] | |
|---|---|---|---|
| | | Mouse | Rat |
| Total recovery (96 hr) | | 93.4 | 95.2 |
| | Urinary metabolites | | |
| XXXII | 3-Hydroxycarbofuran | 9.0 | 6.4 |
| XXXIII | 3-Ketocarbofuran | 11.0 | 9.2 |
| XXXIV | Carbofuran phenol | 42.6 | 39.2 |
| XXXV | 3-Hydroxycarbofuran phenol | 2.0 | 20.1 |
| XXXVI | 3-Ketocarbofuran phenol | 27.5 | 16.4 |
| | Unknown 1 | 2.0 | 3.0 |
| | Unknown 2 | 1.0 | 1.0 |
| | Fecal metabolites | | |
| XXX | PSC | 1.7 | 2.6 |
| XXXII | 3-Hydroxycarbofuran | 0.4 | - |
| XXXIV | Carbofuran phenol | 0.6 | 1.5 |
| XXXV | 3-Hydroxycarbofuran phenol | 0.4 | - |
| XXXVI | 3-Ketocarbofuran phenol | 1.0 | - |
| | Unknown 1 | 0.3 | - |
| | Unknown 2 | 0.4 | 0.6 |
| | Total recovered radioactivity | 100.0 | 100.0 |

[a] Mean from two separate experiments.

Metabolism in House Flies - Results of the metabolism of $^{14}CH_3$-N-PSC in female susceptible house flies ($S_{NAIDM}$ strain) treated topically at a dosage of 10 µg/g are presented in Tables 11 and 12. Presented are data for the distribution of radioactivity and the nature of the various metabolites recovered 4 hr after treatment. Recovery of the applied radioactivity in several experiments ranged from 88 to 95%

The data in Table 11 reveal that PSC is rapidly absorbed by the flies. At zero time, i.e., immediately after treatment, an external methanol-acetone rinse returned 84% of the applied radioactivity. This decreased to 46, 35 and 21% after 1, 2, and 4 hr, respectively.

Detailed analysis for metabolic products was made after 4 hr, at which time most of the flies were in a knockdown state and exhibited typical cholinergic symptoms of intoxication. The flask rinse contained unchanged PSC (5.7%) which was likely physically rubbed from the house fly pronota. An additional amount of water soluble material was observed (2.3%) which was not characterized. The external fly rinse fraction, representing approximately 20% of the total applied radioactivity, contained a lesser amount of PSC (1.9%) and three metabolites, i.e., 3-hydroxycarbofuran (5.3%), N-hydroxymethylcarbofuran (6.1%), and a polar unknown (4.6%) which did not migrate in the tlc systems employed. The remaining 4.6% in the fly rinse fraction was water soluble even after acid treatment.

Approximately 70% of the total recovered radioactivity was present in the fly extract after homogenization of rinsed flies with methanol-acetone solvent, of which approximately one-third partitioned in favor of chloroform over water. The chloroform fraction of the fly homogenate contained PSC (9.9%) and carbofuran (13.5%), the latter undoubtedly being the agent responsible for intoxication owing to its strong anticholinesterase activity. The principal metabolites which were extractable into chloroform after mild acid treatment of the aqueous phase were 3-hydroxycarbofuran (22.7%), 3-ketocarbofuran (7.1%), N-hydroxymethylcarbofuran (6.5%) and a polar unknown (3.5%) which stayed at the origin of the tlc plate. An additional 7.8% of the radioactivity remained in the aqueous phase even after acid treatment.

A summation of the amounts of individual metabolites recovered from the house fly, i.e., sum of internal extract, flask and fly rinse, is presented in Table 12. Examination of the data shows that at least 78% of the recovered radioactivity (91%) was in a form of a metabolite in which the carbamate ester linkage was intact. This is in striking contrast to the spectrum of metabolites which were isolated from rodents (cf Table 10) where 72-76% of the radioactivity was in the form of hydrolytic products, e.g., carbofuran phenol, 3-ketocarbofuran phenol, and 3-hydroxycarbofuran phenol. Thus, the results point to a marked difference in the pathways for the metabolism of PSC in the house fly and mouse or rat. This difference in metabolism is in agreement with the selectivity of PSC toward insects and its relative safety to mammals.

Table 11. METABOLITES PRESENT IN EACH EXTRACT FRACTION FROM FEMALE HOUSE FLIES 4 HOURS AFTER TOPICAL APPLICATION (8 μC/G) OF $^{14}CH_3$-N-PSC

| | | | Per cent |
|---|---|---|---|
| Total recovery[a] | | | 91.0 |
| | Flask rinse | | |
| XXX | PSC | | 5.7 |
| | Water soluble (unhydrolyzed) | | 2.3 |
| | | Total | 8.0 |
| | Fly rinse | | |
| XXX | PSC | | 1.9 |
| XXXII | 3-Hydroxycarbofuran | | 5.3 |
| XXXVII | *N*-Hydroxymethylcarbofuran[b] | | 6.1 |
| | Unknown | | 3.1 |
| | Water soluble | | 4.6 |
| | | Total | 21.0 |
| | Fly extract | | |
| XXX | PSC | | 9.9 |
| XXXI | Carbofuran | | 13.5 |
| XXXII | 3-Hydroxycarbofuran | | 22.7 |
| XXXIII | 3-Ketocarbofuran | | 7.1 |
| XXXVII | *N*-Hydroxymethylcarbofuran | | 6.5 |
| | Unknown | | 3.5 |
| | Water soluble | | 7.8 |
| | | Total | 71.0 |
| Total recovered radioactivity | | | 100.0 |

---

[a] Based upon amount administered.

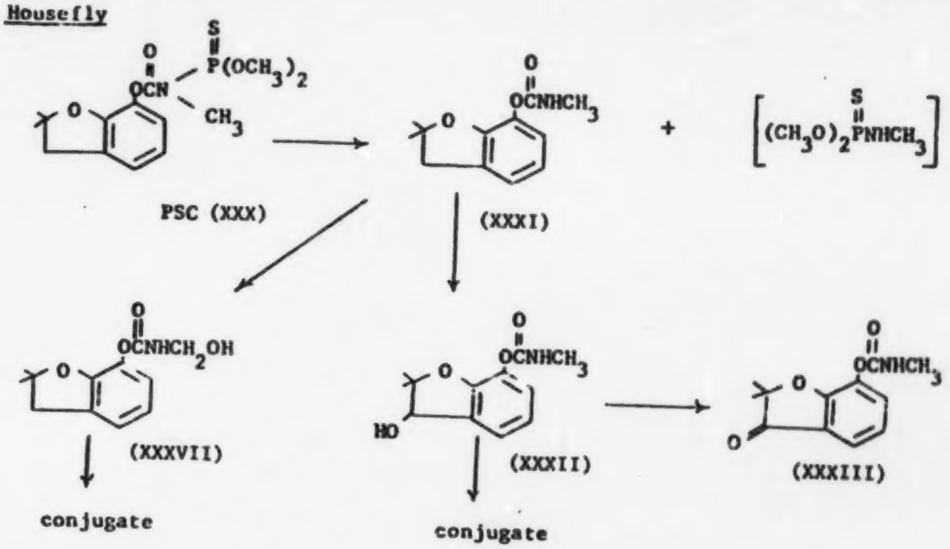
[b] $R_f$ ether-hexane, 0.14; $R_f$ $CH_2Cl_2$-$CH_3CN$, 0.10.

Table 12. SUMMARY OF METABOLITES RECOVERED FROM HOUSE FLIES TREATED WITH $C^{14}H_3$-N-PSC

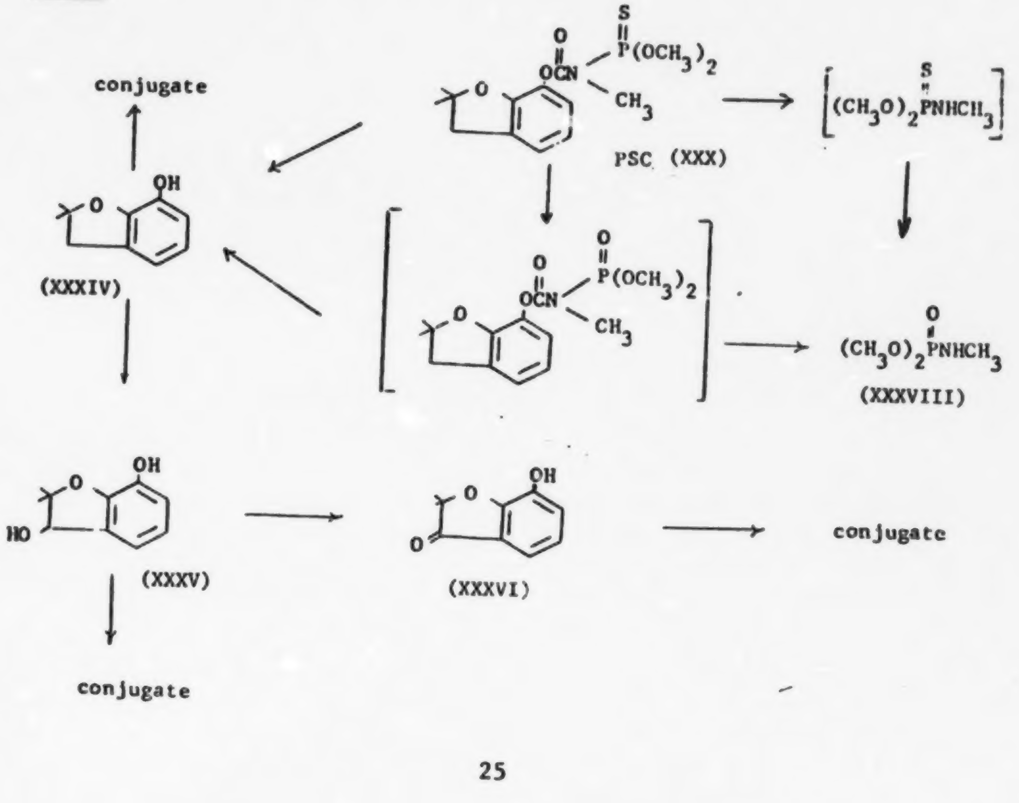
| | | Per cent |
|---|---|---|
| XXX | PSC | 17.5 |
| XXXI | Carbofuran | 13.5 |
| XXXII | 3-Hydroxycarbofuran | 28.0 |
| XXXIII | 3-Ketocarbofuran | 7.1 |
| XXXVII | N-Hydroxymethylcarbofuran | 12.6 |
| | Unknowns | 6.6 |
| | Unextractable metabolites | 14.7 |

Conclusion - The selective toxicity of PSC is clearly associated with fundamentally different pathways of metabolism in house flies and in rats and mice. The basis for selectivity is shown in the metabolic schemes given below

Housefly


S
P(OCH3)2
O
OCN
CH3
PSC (XXX)
O
OCNHCH3
(XXXI)
+
S
(CH3O)2PNHCH3
O
OCNHCH2OH
(XXXVII)
conjugate
O
OCNHCH3
HO
(XXXII)
conjugate
O
OCNHCH3
O
(XXXIII)

Rodent


conjugate
S
O
P(OCH3)2
OCN
CH3
PSC (XXX)
S
(CH3O)2PNHCH3
OH
(XXXIV)
O
O
P(OCH3)2
OCN
CH3
O
(CH3O)2PNHCH3
(XXXVIII)
OH
HO
(XXXV)
conjugate
OH
O
(XXXVI)
conjugate

In house flies, cleavage of the P-N in the phosphoramide moiety occurs resulting in the *in vivo* liberation of the toxic carbamate carbofuran and other intact carbamate metabolites. Thus, the metabolic activation of PSC which occurs in house flies is analogous to the activation of *N*-acetyl Zectran in the spruce budworm[2] and *N*-(2-toluenesulfenyl) carbofuran in house flies[3].

In rodents, PSC is largely degraded to nontoxic phenolic products by hydrolytic and oxidative reactions. It is not certain whether desulfuration precedes or follows hydrolysis of the carbamate ester moiety. Conceivably, PSC may undergo desulfuration first to give the dimethoxyphosphinyl derivative (in brackets) which subsequently is hydrolyzed to carbofuran phenol and dimethyl *N*-methylphosphoramidate. Alternatively, hydrolysis may occur first to yield *O*,*O*-dimethyl *N*-methylphosphoramidothioate which is then converted to the phosphoramidate. *O*,*O*-Dimethyl *N*-methylphosphoramidothioate partitions into chloroform from water and, therefore, it, as well as PSC, probably is sufficiently lipophilic to act as a substrate for mixed function oxidase systems which undoubtedly carry out the desulfuration reaction. It should be pointed out that both of the intermediates in brackets were synthesized by independent methods and neither of these compounds corresponded by tlc to any of the unknown metabolites.

Phenolic hydrolysis products of PSC constituted approximately 75% of the radioactivity recovered from both the mouse and rat after administration of 100 mg/kg $^{14}$C-ring PSC. The relative amounts of hydrolysis products are greater than that recovered from mice (24% phenolics) treated with carbofuran at the $LD_{50}$ dosage of 2 mg/kg, but are similar to that recovered from rats (73% phenolics) also treated with 2 mg/kg carbofuran (one-half $LD_{50}$ level). Since the amount of PSC used in this study, even on a molar basis, is much larger than carbofuran used in earlier studies, the large quantities of phenolic products from PSC suggest that the dimethoxyphosphinothioyl moiety in PSC provides an opportunity for the rat and mouse to carry out metabolic reactions leading to detoxified products.

## 4. Metabolism of 2,2-Dimethyl-2,3-Dihydrobenzofuranyl-7 N-Methyl-N-(2-Toluenesulfenyl)carbamate in the House Fly and White Mouse.

*Introduction* - The preceding section summarized results on the comparative metabolism of PSC in the house fly, rat, and mouse. As in the case of dialkoxyphosphinothioyl derivatives of toxic carbamate insecticides, derivatives produced by aryl- or alkylsulfenylation also are relatively safe to mammals and, further, often exhibit improved insecticidal activity compared to the parent carbamate. In continuation of our efforts in seeking basic information for the design of selectively toxic compounds, a study of the comparative metabolism of 2,2-dimethyl-2,3-dihydrobenzofuranyl-7 *N*-methyl-*N*-(2-toluenesulfenyl)carbamate [XXXIX, or *N*-(2-toluenesulfenyl)carbofuran] in the house fly and white mouse was conducted. Compared to carbofuran (XXXI), XXXIX, with an oral $LD_{50}$ of

100 mg/kg, is 50-fold less toxic to the white mouse and yet is almost twice as toxic to the common house fly.

Metabolism in the White Mouse - Studies were conducted with $^{14}C$ carbonyl and ring-labeled N-(2-toluenesulfenyl)carbofuran (XXXIX) administered orally at a dosage of 15 mg/kg. XXXIX was metabolized rapidly in the white mouse with by far the major portion of the administered radioactivity appearing in the urine within 24 hours. Little, if any, radioactivity appeared in the urine after this time. For each label the ultimate recovery of radioactivity was at least 90% of the dose administered.

Table 13 summarizes data for the distribution, identity, and relative amounts of the various metabolites found after treatment of the mouse with a single dose of XXXIX at 15 mg/kg. By far, the greatest amount of radioactivity was present in the urine as water-soluble conjugates. The feces contained only 3.2 and 5.6% of the recovered radioactivity for ring-$^{14}C$ and carbonyl-$^{14}C$ labels, respectively. The identities of the various metabolites were confirmed by cochromatography in at least four different solvent systems and in one case by mass spectroscopy.

The low recovery of XXXIX in these experiments indicates that the mouse is able to metabolize this compound effectively. Further, the relatively low amount of N-sulfenyl metabolites dectected suggests that N-S bond cleavage occurs to a significant extent. By far, the greatest portion of the recovered radioactivity was in the form of conjugated metabolites of carbonfuran as found in previous studies[4,5,6].

Metabolites detected in this investigation corresponded closely to those found in earlier studies of carbofuran metabolism in the mouse and rat[4,5] although significant quantitative differences were found. For example, the 3-ketocarbofuran from the rat[6], was much less evident in the present study. Although 3-hydroxycarbofuran (XXXII) is a major metabolite of XXXIX from the mouse and of carbofuran from both the mouse and rat[4,5], the dihydroxy metabolite (XLII) in rat urine comprised only 5.3% of the applied dose of carbofuran but was present to a much larger degree (26.8-31.0%) in the present study. These quantitative differences in the metabolism of XXXIX and carbofuran probably are responsible, at least in part, for the reduced toxicity of XXXIX to the mouse.

The three phenolic metabolites XXXIV, XXXV and XXXVI were each present in significant amounts as conjugates in mouse urine. Together, these metabolites account for almost one-third of administered ring labeled XXXIX. The reasonable agreement between the total amount of expired $^{14}C\text{-}CO_2$ trapped suggests that the latter can serve as a good indicator for metabolism of XXXIX by the mouse.

In order to compare further the metabolism of XXXIX with that of carbofuran under similar conditions, additional data for the expiration of $^{14}CO_2$ was obtained. Mice were treated individually with 0.20 mg/kg each of carbonyl labeled XXXIX and carbofuran and the expiration of

Table 13. METABOLIC PRODUCTS RECOVERED FROM THE WHITE MOUSE AFTER ORAL ADMINISTRATION OF 15 MG/KG N-(2-TOLUENESULFENYL) CARBOFURAN (XXXIV)

| | | Ring label (%) | CO label (%) |
|---|---|---|---|
| Total recovery | | 98.7 | 90.0 |
| **Urinary metabolites** | | | |
| | a. Organo-soluble[a] | | |
| XLI | N-(2-Toluenesulfenyl) 3-keto-carbofuran | - | 0.2 |
| XXXII | 3-Hydroxycarbofuran | 1.0 | 3.3 |
| XXXIII | 3-Ketocarbofuran | 0.3 | 0.3 |
| XLII | 3-Hydroxy-N-$CH_2OH$-carbofuran | 5.9 | 10.6 |
| | b. Water-soluble conjugates | | |
| XXXIV | Carbofuran phenol | 12.9 | - |
| XXXV | 3-Hydroxycarbofuran phenol | 11.7 | - |
| XXXVI | 3-Ketocarbofuran phenol | 4.5 | - |
| XXXII | 3-Hydroxycarbofuran | 29.2 | 13.3 |
| XLII | 3-Hydroxy-N-$CH_2OH$-carbofuran | 20.9 | 20.4 |
| | Unextractables | 10.4 | 11.8 |
| **Fecal metabolites** | | | |
| XXXIX | N-(2-Toluenesulfenyl) carbofuran | 0.5 | 0.3 |
| XL | N-(2-Toluenesulfinyl) carbofuran | - | 1.4 |
| XLI | N-(2-Toluenesulfenyl) 3-keto carbofuran | - | 2.7 |
| XXXI | Carbofuran | 1.0 | - |
| | Unextractables | 1.7 | 1.2 |
| | $CO_2$[a] | - | 34.5 |
| | Total | 100.0 | 100.0 |

[a] Based on total recovered radioactivity.

$^{14}CO_2$ was monitored for 24 hr. The results showed that at this lower dose, XXXIX is metabolized more rapidly and to a greater extent than is carbofuran. In the 4 hr following treatment, the mouse treated with XXXIX produced $^{14}CO_2$ at twice the rate of a mouse similarly treated with carbofuran. After 6 hr, expiration of $CO_2$ occurred slowly and finally yielded 41 and 36% of the administered radioactivity for XXXIX and carbofuran, respectively, in 24 hr. The enhanced susceptibility of the N-arylsulfenyl carbamate to this manner of detoxication is doubtlessly a significant factor contributing to the reduced toxicity of XXXIX to mice.

Metabolism in House Flies - Results of metabolism studies with susceptible $S_{NAIDM}$ house flies are presented in Table 14. In each case the flies were treated with radiolabeled XXXIX at 2.85 μg/g. The distribution of radioactivity found in the various fractions 3 hours after treatment is shown in the table. Recoveries of applied radioactivity of greater than 90% were achieved.

All fractions were analyzed by tlc. The conjugated metabolites recovered from the internal extracts were subjected to enzymatic hydrolysis before analysis. The conjugated material recovered from the cage rinse was not analyzed further.

Significant amounts of XXXIX, carbofuran (XXXI) and 3-hydroxycarbofuran (XXXII) were recovered. 3-Ketocarbofuran (XXXIII) was detected only in the hydrolysate of the internal fraction. Almost certainly, this material results from oxidation of XXXII which was also present. Oxidation of XXXII to XXXIII on tlc plates was noted during the identification procedures. Only trace amounts of the dihydroxy metabolite (XLII) were recovered.

Table 15 summarizes the total amounts of the various metabolites identified in all fractions obtained from the house flies. XXXIX was recovered only from the two external fractions (cage and body rinse) and none was detected internally in the fly. The relatively low recovery of this material, after only 3 hr suggests that it is rapidly metabolized by the fly. The detection of small amounts of carbofuran in both of the external fractions further suggests rapid formation of this carbamate from the applied compound. The appreciable amounts of carbofuran accumulated internally in the house fly, corresponding to 0.32 μg/g and 0.68 μg/g for the ring and carbonyl lables, respectively, indicate its role as the toxic agent. Such amounts are comparable and probably even greater than the amounts recoverable when house flies are treated in a similar manner with carbofuran itself. For example, 1 hr after treatment at 2.5 μg/g Dorough[5] found 0.31 μg/g of unchanged carbofuran internally. Since carbofuran is most certainly responsible for intoxication, i.e., by inhibition of cholinesterase, the high amounts recovered after treatment with XXXIX are consistent only with the rapid metabolism of this compound to produce lethal amounts of carbofuran in vivo.

Table 14. METABOLIC PRODUCTS RECOVERED FROM SUSCEPTIBLE HOUSE FLIES AFTER TOPICAL APPLICATION OF 2.85 μG/G N-(2-TOLUENESULFENYL) CARBOFURAN (XXXIX)

| | | Ring label (%) | CO label (%) |
|---|---|---|---|
| Total recovery | | 90.4 | 93.7 |
| Analysis[b] | | | |
| | Cage rinse | | |
| XXXIX | N-(2-Toluenesulfenyl) carbofuran | 1.6 | 2.3 |
| XXXII | Carbofuran | 0.6 | 0.5 |
| | Conjugates | 2.5 | 1.5 |
| | Body rinse | | |
| XXXIX | N-(2-Toluenesulfenyl) carbofuran | 26.1 | 22.8 |
| XXXI | Carbofuran | 1.2 | 2.9 |
| XXXII | 3-Hydroxycarbofuran | 5.9 | 7.2 |
| | Internal extracts | | |
| XXXI | Carbofuran | 17.2 | 37.3 |
| XXXII | 3-Hydroxycarbofuran | 8.1 | 9.4 |
| XLII | 3-Hydroxy-N-$CH_2OH$-carbofuran | 1.6 | trace |
| | Conjugates: | | |
| | XXXII 3-Hydroxycarbofuran | 28.2 | 3.5 |
| | XXXIII 3-Ketocarbofuran | 3.4 | 1.4 |
| | XLII 3-Hydroxy-N-$CH_2OH$-carbofuran | 1.2 | 1.3 |
| | Unextractables | 2.4 | 5.3 |
| | $CO_2$ | - | 4.6 |
| | Total | 100.0 | 100.0 |

---

[a] Based on administered radioactivity.

[b] Based on recovered radioactivity.

Table 15. INTERNALLY RECOVERED METABOLITES FROM HOUSE FLIES

| | Ring label (%) | CO label (%) |
|---|---|---|
| Carbofuran | 17.2 | 37.3 |
| 3-Hydroxycarbofuran | 36.3 | 12.9 |
| 3-Ketocarbofuran | 3.4 | 1.4 |
| 3-Hydroxy-*N*-$CH_2OH$-carbofuran | 2.8 | 1.3 |

Supporting evidence for the rapid action of XXXIX was obtained by electrophysiological techniques[7]. The intoxication of house flies treated topically with 0.5 μg of XXXIX was monitored and closely compared to the effects of carbofuran measured under the same conditions Application of XXXIX produced effects indistinguishable from the effects of carbofuran poisoning except that XXXIX acted more rapidly. Intoxication by XXXIX was evident only 6 min after application, whereas carbofuran required 12 min to produce the same physiological response. If carbofuran is the common toxicant, this result leaves little doubt of the rapid absorption and metabolism of XXXIX to carbofuran in house flies.

Toxicology - Toxicological data for various metabolites and derivatives of XXXIX and carbofuran are presented in Table 16. XXXIX and its derivatives are generally more toxic to the house fly, yet are still relatively safer to the mouse than the corresponding carbofuran derivatives. The insecticidal activity of N-(2-toluenesulfenyl) 3-ketocarbofuran as compared with 3-ketocarbofuran is particularly noteworthy. Oxidation of the sulfenamide XXXIX to the sulfinamide reduces insecticidal activity to some degree but, with an oral $LD_{50}$ of 75 mg/kg, the sulfinamide is still reasonably safe to the mouse. Further oxidation to the sulfonamide destroys toxicity to both the insect and mammal.

The appreciable oral toxicities of the carbofuran metabolites are notable and doubtlessly contribute to the high mammalian toxicity of carbofuran.

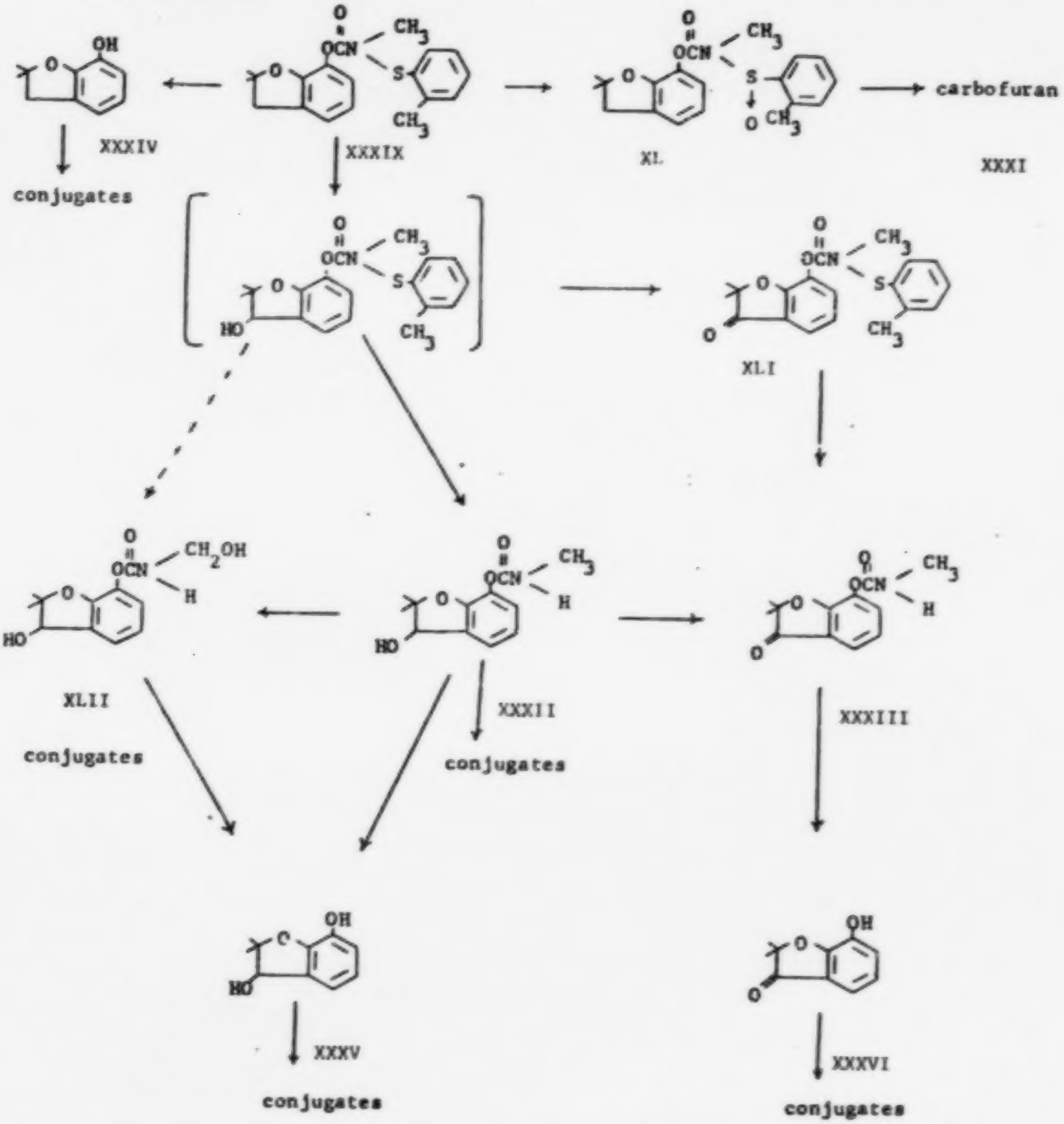
In accord with reduced mammalian toxicities, XXXIX is 19-fold less effective as an inhibitor of bovine erythrocyte acetylcholinesterase (BAChE) than is carbofuran. On the other hand, XXXIX is 76-fold less inhibitory to house fly acetylcholinesterase (HAChE) than is carbofuran and this decreased potency further supports the view that carbofuran, and not XXXIX is the primary toxicant to house flies. Oxidation at the sulfur atom to give the sulfinamide influences anticholinesterase activity but does not greatly affect toxicities. Further oxidation to the sulfonamide destroys toxicity and severely reduces anticholinesterase activity.

N-(2-Toluenesulfenyl) 3-ketocarbofuran is surprisingly almost as effective as an inhibitor of BAChE as is 3-ketocarbofuran, although it is somewhat less active toward HAChE. As expected, the carbofuran metabolites are all effective inhibitors of both enzymes. In summary, the N-substituted-N-methylcarbamates are generally poorer inhibitors of both insect and mammalian acetylcholinesterases, a result which again suggests that processes other than direct enzyme inhibition are responsible for the selective toxicity of XXXIX.

Conclusion - The basis for the selective toxicity of N-(2-toluenesulfenyl) carbofuran (XXXIX) is undoubtedly attributable to different paths of metabolism in the mouse and house fly. Clearly, XXXIX is rapidly absorbed and metabolized in house flies to produce carbofuran by cleavage to the N-S bond. This process probably occurs more slowly in the mouse and thereby provides an opportunity for detoxication processes to occur.

Presented below is the scheme for the metabolism of XXXIX in the mouse.

MOUSE METABOLISM



XXXIV
conjugates
XXXIX
XL
carbofuran
XXXI
XLI
XLII
conjugates
XXXII
conjugates
XXXIII
XXXV
conjugates
XXXVI
conjugates

The primary route of metabolism is a series of detoxication steps and probably proceeds via the key intermediate *N*-(2-toluenesulfenyl) 3-hydroxycarbofuran (in brackets). Although this intermediate was not isolated, evidence for its formation rests with the recovery of significant amounts of *N*-(2-toluenesulfenyl) 3-ketocarbofuran (XLI) and the relatively large amounts of 3-hydroxycarbofuran (XXXII) and the dihydroxycarbofuran (XLII). Although XXXII is a major metabolite of carbofuran in both the rat and mouse[4,5], small amounts of XLII were found only with the rat. The formation of XLII by hydroxylation of *N*-(2-toluenesulfenyl) 3-hydroxycarbofuran and subsequent cleavage of the N-S bond would be consistent with the observed metabolites.

Conjugation mechanisms almost certainly play an important role in the further detoxication of hydroxylated carbofuran metabolites. XXXII is quite toxic to the mouse and is recovered in significant amounts from mouse urine. However, by far the greater proportion of hydroxy metabolites recovered were in the conjugated form. The formation of conjugates would seem, therefore, to provide an important additional pathway in detoxication of XXXIX.

The ability of the mouse to metabolize XXXIX at a rate faster than carbofuran, as estimated by $^{14}CO_2$ respiration studies, may well provide the most important reason for its reduced toxicity to mammals.

The basis for the toxicity of XXXIX to house flies is apparent from the metabolism data. Presented below is the scheme proposed for its metabolism in house flies and it appears that XXXIX is peculiarly suited for liberation of carbofuran in the fly. The recovery of large amounts of carbofuran in the house fly agrees with its role as the toxic agent. The complete absence of the applied compound, or any of the other *N*-sulfenyl derivatives in the *internal fraction*, further implicates carbofuran and not the sulfenamides as the toxicant. The liberation of carbofuran by cleavage of the N-S bond of XXXIX occurs as an activation step in house flies.

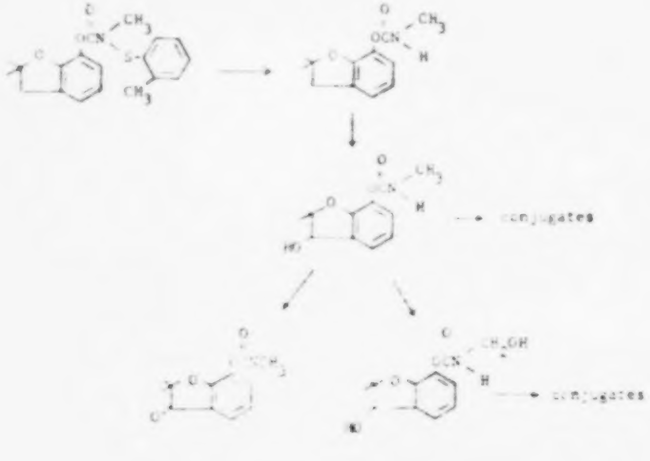


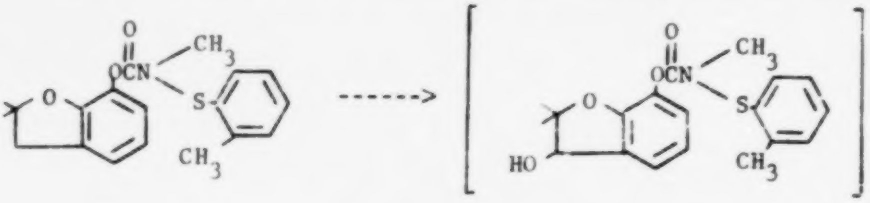
Table 16. TOXICOLOGICAL PROPERTIES OF N-(2-TOLUENESULFENYL) CARBOFURAN (XXXIX) AND ITS CARBAMATE METABOLITES AND DERIVATIVES

| Compound | Anticholinesterase activity BAChE $k_i$ ($M^{-1}$ $min^{-1}$) | HAChE $k_i$ ($M^{-1}$ $min^{-1}$) | Toxicity Mouse oral mg/kg[a] | House-fly µg/g |
|---|---|---|---|---|
| N-(2-Toluenesulfenyl) carbofuran | $1.0 \times 10^5$ | $1.7 \times 10^5$ | 100-25 | 3.2 |
| N-(2-Toluenesulfinyl) carbofuran | $3.7 \times 10^4$ | $3.5 \times 10^5$ | 75 | 9.0 |
| N-(2-Toluenesulfonyl) carbofuran | $2.3 \times 10^3$ | $1.5 \times 10^4$ | >150 | >500 |
| N-(2-Toluenesulfenyl) 3-keto-carbofuran | $2.3 \times 10^5$ | $8.6 \times 10^4$ | 150 | 22.0 |
| Carbofuran | $1.9 \times 10^6$ | $1.3 \times 10^7$ | 2 | 6.7 |
| 3-Hydroxy-carbofuran | $3.2 \times 10^5$ | $1.4 \times 10^6$ | 7[a] | >500 |
| N-Hydroxymethyl-carbofuran | $2.4 \times 10^5$ | $6.3 \times 10^5$ | 30-75[a] | >500 |
| 3-Keto-carbofuran | $3.3 \times 10^5$ | $2.3 \times 10^5$ | 10-30[a] | >500 |
| 5-Hydroxy-carbofuran | $1.2 \times 10^5$ | $1.8 \times 10^6$ | - | - |

[a] Determined in propylene glycol solvent.

## 5. Thiolysis as an Activation Process in N-Sulfenylated Derivatives of Methylcarbamate Esters

Introduction - On the basis of its comparative metabolism in the white mouse and house fly, the selective toxicity of N-(2-toluenesulfenyl) carbofuran (XXXIX) was attributed to differing pathways of metabolism between the mammal and insect. In house flies, lethal quantities of carbofuran were produced in vivo after topical application of XXXIX, while the mouse degraded XXXIX largely to detoxified conjugated products. Owing to the nature of the metabolic products isolated from the mouse, it was postulated that the initial step in the metabolic sequence was the formation of the intermediate N-(2-toluenesulfenyl)-3-hydroxycarbofuran, even though this material was not isolated. A study was conducted in an attempt to establish the intermediacy of N-(2-toluenesulfenyl)-3-hydroxycarbofuran in the metabolic pathway by examining the fate of XXXIX in vitro in subcellular fractions of mouse liver. This section describes the unexpected results which were obtained and focuses on the nature of the cleavage of the N-S bond in sulfenylated carbamate esters.



XXXIX

N-S Bond Cleavage by Biological Tissue - Contrary to expectation, the various subcellular fractions of mouse liver indicated in Table 17 rapidly effected the cleavage of the N-S bond of N-(2-toluenesulfenyl) carbofuran (XXXIX) to generate carbofuran as the principal product. Evidently, XXXIX is stable in tris buffer (pH 7.4) since 100% unchanged material was recovered after a fixed incubation period of 10 min. In the presence of different liver tissue, however, XXXIX was quite unstable, the amount of XXXIX recovered ranging from 0 to 26%. The addition of NADPH cofactor markedly reduced the amount of carbofuran produced owing to oxidation of carbofuran to other metabolic products, e.g., to 3-hydroxycarbofuran. This was particularly apparent with the homogenate and microsomal fractions, providing evidence that each of these fractions consisted of active preparations of mixed function oxidase. Based on the quantity of XXXIX recovered, the supernatant appeared to be more effective in producing carbofuran than the microsomal fraction, although the microsomal preparation after heat denaturation showed the highest N-S bond cleaving activity after 1 hour incubation. The high activity of denatured microsomes suggests that the N-S bond is cleaved by a chemical rather than an enzymatic process.

Table 17. DEGREE OF N-S BOND CLEAVAGE OF *N*-(2-TOLUENESULFENYL) CARBOFURAN (XXXIX) BY SUBCELLULAR FRACTIONS OF MOUSE LIVER AFTER 10 MIN AT 30°C

| Subcellular fraction | Relative amounts of products obtained (%) | | |
|---|---|---|---|
| | XXXIX | Carbofuran | Other |
| Buffer alone | 100 | 0 | 0 |
| Homogenate | 11 | 84 | 5 |
| Homogenate + NADPH | 8 | 52 | 40 |
| Microsome | 26 | 72 | 2 |
| Microsome + NADPH | 22 | 48 | 30 |
| Supernatant | 8 | 91 | 1 |
| Supernatant + NADPH | 9 | 89 | 2 |
| Boiled microsome[a] + NADPH | 0 | 100 | 0 |

[a] Liver microsomal fraction was heated in boiling water for 10 min and incubated with XXXIX and NADPH for 1 hr.

The reported lability of the N-S bond in the fungicide Captan to sulfur nucleophiles[8,9] prompted the examination of the effect of L-cysteine and reduced glutathione. Each of these reagents at $10^{-3}$ M in tris buffer, pH 7.4, quantitatively converted XXXIX into carbofuran under the usual conditions of 10 minute incubation at 30°C. This drew attention to the examination of different biological preparations, including bovine serum albumin (BSA), house fly homogenate, and mouse blood for total thiol titre and its relationship to degree of N-S bond cleavage. The results, summarized in Table 18, indicate a fairly good linear relationship between thiol titre, as expressed by GSH equivalency, and the level of carbofuran produced. Of the biological preparations examined, mouse blood, with a thiol titre equivalent to $3.1 \times 10^{-4}$ M GSH gave 100% carbofuran compared to 29% for BSA whose GSH equivalent was $0.75 \times 10^{-4}$ M.

In the presence of sulfhydryl inhibitors, e.g., N-ethylmaleimide (NEM) and p-chloromercuribenzoate (PCMB), the production of carbofuran was completely inhibited and XXXIX was quantitatively recovered. This suggests that free thiol groups are required in biological tissues to effect N-S bond cleavage.

Kinetic Analysis of N-S Bond Cleavage - The reaction between the fungicide Captan (N-trichloromethylthio-4-cyclohexane-1,2-dicarboxyimide) and Folpet (N-trichloromethylthiophthalimide), each containing an N-S bond, and p-nitrobenzenethiol (NBT) and other thiols has been previously described[9]. Because of the convenience with which NBT may be estimated colorimetrically (410 nm), this reagent was selected for analysis of N-S bond cleavage of sulfenylated methylcarbamates by thiolate nucleophiles. When XXXIX and other sulfenylated methylcarbamates were allowed to stand with NBT, the absorption of the yellow anion decreased rapidly with time, giving as products the methylcarbamate and a mixture of disulfides (cf section on product analysis). For example, the absorption of a buffered solution of $8 \times 10^{-5}$ M NBT and $1.34 \times 10^{-4}$ M XXXIX diminished virtually to zero within a period of 5 min. No detectable decrease in absorption of NBT was noted when carbofuran was substituted for the sulfenylated derivative.

In addition to XXXIX, the N-(2-toluenesulfinyl) (XLI), and N-(2-toluenesulfonyl) (XLII) derivatives of carbofuran also were examined for their reaction with NBT. These derivatives also react with NBT but at reduced rates and in the decreasing order of sulfenyl > sulfinyl >> sulfonyl. The reaction between NBT and the sulfenylated carbamate derivatives proceeded with second-order kinetics and values for the rate constants for a number of different derivatives are summarized in Table 19. As expected, arylsulfenyl derivatives substituted with electron withdrawing substituents, e.g., the halogen and cyano derivatives, were most susceptible to reaction with NBT and an orderly decrease in rate constants was observed as the substituents became less electron withdrawing. The reaction between NBT and the pentachlorobenzenesulfenyl derivative occurred in such a rapid manner that a rate

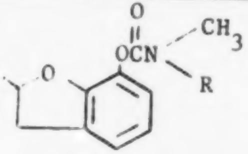
Table 18. RELATION BETWEEN ESTIMATED CONCENTRATION OF FREE THIOL GROUPS IN DIFFERENT BIOLOGICAL TISSUE AND DEGREE OF N-S BOND CLEAVAGE OF N-(2-TOLUENESULFENYL) CARBOFURAN (XXXIX) AFTER 10 MIN AT 30°C

| Tissue or Reagent | Total SH (by O.D.) | SH Equiv. to GSH (M) | XXXIX (%) | Carbofuran (%) |
|---|---|---|---|---|
| $10^{-3}$ M L-Cysteine | - | - | 0 | 100 |
| $10^{-3}$ M GSH | - | - | 0 | 100 |
| 0.1 ml Mouse blood | 4.1 | $3.1 \times 10^{-4}$ | 0 | 100 |
| 0.5 ml 20% Liver homog. | 3.3 | $2.5 \times 10^{-4}$ | 11 | 86[a] |
| 0.5 ml 20% Fly homog. | 1.4 | $1.1 \times 10^{-4}$ | 61 | 39 |
| 50 mg BSA | 1.0 | $7.5 \times 10^{-5}$ | 71 | 29 |
| $10^{-2}$ M NEM in each tissue prep.[b] | - | - | 100 | 0 |
| $10^{-3}$ M PCMB in each tissue prep.[b] | - | - | 100 | 0 |

[a] Approximately 3% of the product was 3-hydroxycarbofuran.

[b] Each tissue preparation was incubated with the indicated concentration of NEM and PCMB for 10 min prior to reaction with XXXIX.

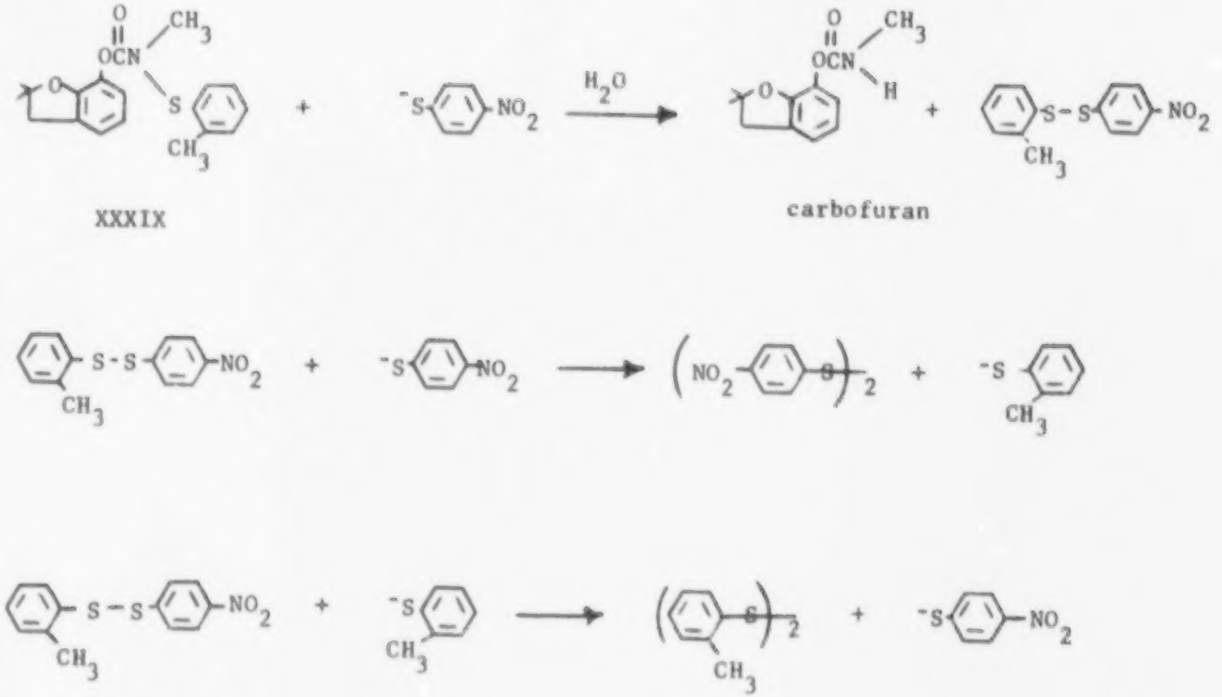
Table 19. SECOND-ORDER RATE CONSTANTS (k) FOR THE CLEAVAGE OF THE N-S BOND IN N-SULFENYLATED DERIVATIVES OF CARBOFURAN BY p-NITROBENZENETHIOL AT pH 7.0 AND 30°C

| R |  | k ($\underline{M}^{-1}$ min$^{-1}$) |
|---|---|---|
| $SCH_3$ | | 6.6 |
| $SC_6H_5$ | | 8.7 |
| $SC_6H_4$-2-$CH_3$ (XXXIX) | | 7.6 |
| $SOC_6H_4$-2-$CH_3$ (XL) | | 2.4 |
| $SO_2C_6H_4$-2-$CH_3$ (XLIII) | | 0.083 |
| $SC_6H_4$-4-$OCH_3$ | | 7.1 |
| $SC_6H_4$-4-t-butyl | | 7.6 |
| $SC_6H_4$-4-Cl | | 24.0 |
| $SC_6H_4$-4-Br | | 27.0 |
| $SC_6H_4$-4-CN | | 35.0 |
| S-2-naphthyl | | 11.3 |
| $SC_6Cl_5$ | | too rapid to measure |
| S-2-(1,3-benzothiazolyl) | | too rapid to measure |

constant could not be determined. Relatively little difference was noted between the unsubstituted benzenesulfenyl derivative and those containing electron donating substituents.

A comparison of the rate constants for N-S bond cleavage and the toxicological properties of a limited number of carbofuran derivatives is made in Table 20. The data show that the derivatives were all significantly less effective in inhibiting both bovine erythrocyte (BAChE) and house fly-head (HAChE) acetylcholinesterase than carbofuran. While a direct relationship between the rates of N-S bond cleavage and toxicity to the house fly and white mouse is not apparent, evidently rapid N-S bond cleavage is required for insecticidal activity. The poor toxicity of the N-(2-toluenesulfonyl) derivative is consistent with its poor anticholinesterase activity and resistance to N-S bond cleavage. This suggests that sulfonyl derivatives containing a more labile N-S bond should be examined for insecticidal activity and work along this line is in progress.

Product Analysis - The experimental condition employed for the analysis of products obtained from the reaction between XXXIX and NBT was similar to that for the kinetic studies except proportionately large quantities of reactants were used. For convenience of analysis, nonradioactive XXXIX was charged with a small amount of $^{14}C$ carbonyl label. The reaction proceeded rapidly and carbofuran was recovered quantitatively after 5 minute reaction, along with a mixture of disulfides. The products were separated and identified by cochromatography (tlc) as (2-tolyl) disulfide, 2-tolyl-4'-nitrophenyl disulfide, bis-(4-nitrophenyl) disulfide, and carbofuran. Of the three disulfides recovered, approximately 80% was 2-tolyl-4'-nitrophenyl disulfide, each of the other two being obtained in about 10%. Based on the products observed, the following equations reasonably depict the reactions which take place.


XXXIX

carbofuran

(1)

(2)

(3)

Table 20. COMPARISON OF RATES OF N-S BOND CLEAVAGE (k) AND TOXICOLOGICAL PROPERTIES OF N-SUBSTITUTED CARBOFURAN DERIVATIVES

| Compound | Anticholinesterase activity $k_i$ ($M^{-1}$ $min^{-1}$) BAChE | HAChE | Toxicity, $LD_{50}$ (mg/kg) Mouse oral | House fly | N-S bond k ($M^{-1}$ $min^{-1}$) |
|---|---|---|---|---|---|
| Carbofuran | $1.9 \times 10^6$ | $1.3 \times 10^7$ | 2 | 6.7 | - |
| N-Benzenesulfenyl-carbofuran | $8.1 \times 10^5$ | $9.8 \times 10^5$ | 25-50 | 72.5 | $8.7 \times 10^3$ |
| N-(p-t-Butylbenzenesulfenyl) carbofuran | $7.8 \times 10^4$ | $6.7 \times 10^5$ | 75 | 2.7 | $7.6 \times 10^3$ |
| N-(2-Toluenesulfenyl) carbofuran | $1.0 \times 10^5$ | $1.7 \times 10^5$ | 100-125 | 3.2 | $7.6 \times 10^3$ |
| N-(2-Toluenesulfinyl) carbofuran | $3.7 \times 10^4$ | $3.5 \times 10^5$ | 75 | 9 | $2.4 \times 10^3$ |
| N-(2-Toluenesulfonyl) carbofuran | $2.3 \times 10^3$ | $1.5 \times 10^4$ | >150 | >500 | $8.3 \times 10$ |

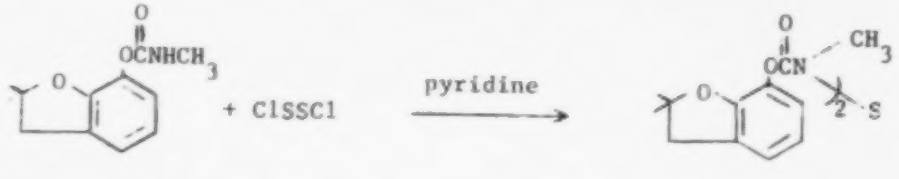
In addition to reactions 1-3, all three disulfides also may result from atmospheric oxidation since the experiments were not conducted under anaerobic conditions. Although the overall process is probably quite complex, the reaction depicted by equation 1 is undoubtedly of major significance owing to the preponderance of carbofuran and the unsymmetrical disulfide as products and the second order nature of the reaction.

Conclusion - The results described in section 4 revealed that the toxicity of *N*-(2-toluenesulfenyl) carbofuran (XXXIX), was attributable to *in vivo* conversion of XXXIX, a weak anticholinesterase, to the potent anticholinesterase carbofuran. The present study indicates that this activation process is nonenzymatic and probably involves thiolytic cleavage of the N-S bond. All tissues, including subcellular fractions of mouse liver, heparinized mouse blood, commercial protein, and house fly homogenate, as well as thiol reagents, readily effected the cleavage of the N-S bond. In limited studies, this was also found to be true with homogenates of mouse stomach and intestine. The fact that no less activity was observed with the heat denatured microsomal fraction of mouse liver and high activity was observed with the supernatant virtually eliminates involvement of oxidase enzymes in the activation process. Further, the inhibitory action of such sulfhydryl inhibitors as NEM and PCMB strongly suggests that thiol residues in biological tissue are involved in the generation of the carbamate from the sulfenylated derivative.

Although the results of this study provide insight into the nature of the alteration reactions which XXXIX and other sulfenylated carbamates undergo in mammals, they are, however, contrary to the rationale proposed for the selective toxicity of these materials. While toxicity to insects may be explained on the basis of *in vivo* thiolysis of the N-S bond to generate the toxic methylcarbamate, the same explanation argues against reduced mammalian toxicity of sulfenylated carbamates owing to the ubiquitous nature of sulfhydryl groups in mammalian tissue. Since the results show that carbofuran is rapidly generated from XXXIX *in vitro* by a variety of mammalian tissue, the much lower mammalian toxicity of XXXIX compared to carbofuran is difficult to explain, particularly since the cleavage of the N-S bond appears to be a nonenzymatic process. It should be reiterated that in the *in vivo* study of XXXIX in the mouse, virtually no carbofuran was recovered and, further, about 90% of the metabolites were in the form of water soluble conjugates of hydroxylated carbofuran or the phenol. However, the facility with which various mammalian tissues are able to catalyze the cleavage of the N-S bond is disturbing and further work is required to explain the discrepancy between *in vivo* and *in vitro* results.

## 6. Selective Toxicity of *N*-Substituted Biscarbamoyl Sulfides.

Introduction - In a separate study, it was demonstrated that when 2,2-dimethyl-2,3-dihydrobenzofuranyl *N*-methyl-*N*-(dimethoxyphosphinothioyl) carbamate (XXX or PSC) was treated with *m*-chloroperbenzoic acid, the principal products observed are the expected desulfuration product 2,2-dimethyl-2,3-benzofuranyl-7 *N*-methyl-*N*-(dimethoxyphosphinyl) carbamate (XLIV) and an unexpected rearrangement product, the corresponding *N*-(dimethoxyphosphinylthio)carbamate (XLV) (cf part F-1) XLV was unstable on the plates and decomposed to carbofuran (XXXI) and to the *N*-substituted biscarbofuran disulfide (XLV). In an attempt to prepare XLV by an independent method, 2 moles of carbofuran was reacted with 1 mole of sulfur monochloride in pyridine solvent. This reaction, however, did not produce XLV but gave the biscarbamoyl sulfide (XLVI) as the principal product according to the equation below.



XXXI

XLVI

To our surprise, XLVI, although much less toxic to the white mouse, was still highly insecticidal, comparable in activity to carbofuran. Therefore, other *N*-substituted biscarbamoyl sulfides were prepared and examined. The following section is concerned with the toxicological properties of these compounds, with particular emphasis on their selective toxicity.

Insecticidal Activity - Toxicological properties of the various *N*-substituted biscarbamoyl sulfides are presented in Table 21, along with those of the parent methylcarbamates. From examination of the data in the table, it is apparent that the *N*-substituted biscarbamoyl sulfides retained much of the insecticidal activity exhibited by the corresponding methylcarbamate esters. Although all of the biscarbamoyl sulfides, used alone or in combination with piperonyl butoxide(PB), were slightly less toxic to house flies compared to the methylcarbamates, they were, however, significantly more toxic to mosquito larvae. Except for bisaldicarb sulfide (LIV), a five- to eightfold increase in larvicidal activity was observed with the biscarbamoyl sulfide.

Since aldicarb (LIII) is used primarily as a systemic insecticide, the corresponding bisaldicarb sulfide (LIV) also was examined for systemic activity in the cotton plant. Laboratory tests against the cotton aphid (*Aphis gossypii*), perforator (*Bucculatrix thurberiella*), mite (*Tetranychus* *cinnabarinus*), and salt marsh caterpillar (*Estigmene acrea*) revealed that LIV applied to soil containing cotton plants was significantly more effective in controlling the first three pests than aldicarb. For example, at the approximate dosage of 1.2 lb of actual material per acre, LIV gave virtually complete control of cotton aphids and perforator for over 21 weeks, as compared to 15 weeks for aldicarb. Aldicarb and LIV were about equal in their activity against the salt marsh caterpillar.

Biscarbofuran sulfide (XLVI) also was examined as a cotton systemic insecticide and although it was outstandingly effective in controlling the cotton leaf perforator, giving 100% control for more than 20 weeks, it was almost totally ineffective against the other pests.

*Mouse Toxicity* - The oral mouse toxicity data presented in Table 21 show that compared to the original methylcarbamates, the *N*-substituted biscarbamoyl sulfides are in most cases substantially less toxic to the white mouse. Depending on the compound, the biscarbamoyl sulfides were from 5- to 50-fold less toxic to the mouse than the parent methylcarbamate. The largest difference (50-fold) in mouse toxicity was observed between carbofuran (XXXI) and biscarbofuran sulfide (XLVI). Bisaldicarb sulfide (LIV) was approximately fivefold less toxic to mice than aldicarb (LIII). In general, improvement in mouse toxicity by conversion of the parent carbamates to the biscarbamoyl sulfides paralleled results obtained in other related derivatization studies.

*Anticholinesterase Activity* - Bimolecular rate constants ($k_i$) for the inhibition of house fly-head (HAChE) and bovine erythrocyte (BAChE) acetylcholinesterase by the methylcarbamate insecticides and *N*-substituted biscarbamoyl sulfides are presented in Table 21. The data clearly indicate that on an overall basis the biscarbamoyl sulfides are less effective anticholinesterases than the parent methylcarbamates against both bovine erythrocyte and house fly-head AChE. Differences in anticholinesterase activity between respective biscarbamoyl sulfides and methylcarbamates were quite variable, ranging from 4.7-fold for aldicarb-bisaldicarb sulfide (LIII:LIV) against BAChE to 141-fold for carbofuran-biscarbofuran sulfide (XXXI:XLVI) against HAChE.

In the case of BAChE, i.e., an AChE from a mammalian source, the reduction in anticholinesterase activity of the biscarbamoyl sulfide compared to the methylcarbamate is more or less in agreement with the reduction in mouse toxicity (cf Table 22). Although probably fortuitous, the correlation between mouse toxicity and BAChE inhibition by aldicarb (LIII) and bisaldicarb sulfide (LIV) is quite good. Correlation between the other pairs, particularly XLIX:L and LI:LII, however, is much less satisfactory.

On the other hand, differences in inhibition between the methylcarbamate-biscarbamoyl sulfide pairs against HAChE were far greater than their respective relative toxicities to house flies. This suggests that factors other than cholinesterase inhibition must be considered to

Table 21. TOXICOLOGICAL PROPERTIES OF INSECTICIDAL METHYLCARBAMATE ESTERS AND THEIR CORRESPONDING N-SUBSTITUTED BIS-CARBAMOYL SULFIDES

| | Compound | $LD_{50}$ (mg/kg) | | | | Anticholinesterase activity $k_i$ ($M^{-1}$ $min^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | House fly | | Mosquito larvae | Mouse | Bovine erythrocyte | Fly-head |
| | | Alone | +P.B.[a] | | | | |
| XXXI | Carbofuran | 6.7 | 0.9 | 0.052 | 2 | $1.9 \times 10^6$ | $1.3 \times 10^7$ |
| XLVI | *Bis*-carbofuran sulfide | 19 | 1.2 | 0.007 | 50-100 | $2.5 \times 10^4$ | $9.2 \times 10^4$ |
| XLVII | Carbaryl | >500 | 12.5 | 1.0 | 560[b] | $4.0 \times 10^4$ | $4.9 \times 10^5$ |
| XLVIII | *Bis*-carbaryl sulfide | >500 | 42.0 | 0.22 | -[c] | $6.1 \times 10^3$ | $5.4 \times 10^4$ |
| XLIX | 3-Isopropylphenyl methylcarbamate (MIP) | 41 | 1.6 | 0.038 | 16 | $7.5 \times 10^5$ | $7.7 \times 10^5$ |
| L | *Bis*-MIP sulfide | 85 | 4.6 | 0.0056 | 200 | $2.7 \times 10^4$ | $2.3 \times 10^4$ |
| LI | Propoxur | 22 | 0.9 | 0.33 | 24 | $4.3 \times 10^4$ | $1.2 \times 10^6$ |
| LII | *Bis*-propoxur sulfide | 35 | 2.5 | 0.041 | 700 | $4.6 \times 10^3$ | $2.8 \times 10^4$ |
| LIII | Aldicarb | 5.5 | 1.0 | 0.16 | 0.3-0.5 | $3.5 \times 10^4$ | $2.0 \times 10^4$ |
| LIV | *Bis*-aldicarb sulfide | 8.5 | 3.2 | 0.17 | 1.6-2.5 | $7.4 \times 10^3$ | $2.1 \times 10^3$ |

[a] Piperonyl butoxide (P.B.) was applied at a constant dose of 40 μg/fly in combination with varying doses of insecticide.

[b] Toxicity to the rat.

[c] Compound was too insoluble in olive oil or propylene glycol for accurate evaluation.

Table 22. RELATIVE VALUES FOR THE INHIBITION OF BAChE AND HAChE AND TOXICITY TO THE MOUSE AND HOUSE FLY FOR THE INDICATED PAIRS OF METHYLCARBAMATE AND BIS-CARBAMOYL SULFIDES

| Compounds | BAChE $k_i$ ratio | Mouse relative toxicity | HAChE $k_i$ ratio | House fly relative toxicity |
|---|---|---|---|---|
| XXXI/XLVI | 76 | 25-50 | 141 | 2.8 |
| XLIX/L | 28 | 13 | 33 | 2.1 |
| LI/LII | 9.3 | 29 | 42 | 1.5 |
| LIII/LIV | 5.2 | 4.7 | 9.5 | 1.5 |

account for the comparatively high insecticidal activity of the biscarbamoyl sulfides relative to their anticholinesterase activity. This is particularly true in connection with mosquito larvae where the biscarbamoyl sulfides are observed to be more toxic than the methylcarbamates.

*Conclusion* - The results show that the conversion of insecticidal methylcarbamate esters to the corresponding biscarbamoylsulfides by treatment with sulfur monochloride in pyridine produced compounds with significantly improved mammalian toxicity while still retaining insecticidal activity. By analogy with the derivatized methylcarbamates reported earlier (Parts B-3 and B-4), the favorable toxicological properties of the biscarbamoyl sulfides may be attributed to opportunities which derivatization may provide in allowing additional metabolic detoxication and intoxication processes to take place in mammals and insects. Although reduced mammalian toxicity of the biscarbamoyl sulfides may be accounted for in part by their lower anticholinesterase activity, it is quite probable that they are detoxified before large amounts of the parent methylcarbamate are produced *in vivo* owing to the unlikelihood that a molecule such as XLVI will pass through the mouse unchanged.

## 7. Metabolism of O,O-Dimethyl S-[α-(Carboethoxy)benzyl] Phosphorodithioate (Phenthoate) in the White Mouse and House Flies

*Introduction* - Phenthoate or *O,O*-dimethyl *S*-[α-(carboethoxy)benzyl phosphorodithoate (LV) is a relatively new organophosphorus insecticide of low mammalian toxicity. Its rat oral $LD_{50}$ is reported to be 4728 mg/kg[10] a value which places it approximately 950-fold less toxic to the rat than to the common house fly ($LD_{50}$ 5 mg/kg). Because of its overall high insecticidal activity and low mammalian toxicity, a study of the comparative metabolism of phenthoate in mammals and insects was carried out to determine the underlying basis for the selective properties of this compound.

*Metabolism in the White Mouse* - Two different radiolabels, $^{14}C$ ring-labeled phenthoate (LV) and $^{32}P$ phenthoate were used. $^{32}P$ phenthoate was administered orally to mice at the dosages of 30 and 161 mg/kg, and $^{14}C$ phenthoate at 17 and 161 mg/kg.

Phenthoate is metabolized rapidly in the white mouse with the major portion of the administered radioactivity appearing in the urine within 24 hours after treatment. Table 23 summarizes data for the distribution of excreted radioactivity 12 and 24 hours following treatment with different single doses of $^{32}P$ or $^{14}C$ phenthoate. For the 24-hour period, the total recovery of radioactivity ranged from 85-100%, of which 85-94% was present in the urine in the form of water soluble materials. The feces contained approximately 5-13% of the administered radioactivity and the fraction present in the feces appeared to be significantly higher at the high dosage (161 mg/kg) than at the lower dosages.

Table 23. CUMULATIVE DISTRIBUTION $^{14}$C- OR $^{32}$P-LABELED PHENTHOATE (LV) IN THE WHITE MOUSE RECEIVING A SINGLE INDICATED DOSE

| Dose (mg/kg) | Time (hours) | % of Administered dose | | | |
|---|---|---|---|---|---|
| | | Urine | | | |
| | | Aqueous | Organic | Feces | Total |
| **$^{32}$P Phenthoate** | | | | | |
| 30 | 12 | 66.5 | 16.4 | 5.0 | 87.9 |
| | 24 | 70.2 | 16.5 | 5.3 | 92.0 |
| 161 | 12 | 52.3 | 7.8 | 8.1 | 68.2 |
| | 24 | 64.5 | 12.3 | 8.2 | 85.0 |
| **$^{14}$C Phenthoate** | | | | | |
| 17 | 12 | 61.0 | 14.6 | 3.8 | 79.4 |
| | 24 | 78.6 | 15.6 | 7.6 | 101.8 |
| 161 | 12 | 39.7 | 14.7 | 11.7 | 66.1 |
| | 24 | 56.2 | 20.6 | 13.0 | 89.8 |

Identification of the various metabolites was achieved by co-chromatography with independently synthesized model metabolites in at least four different solvent systems. Table 24 gives the identity and relative amounts of the metabolites detected in the urine and feces of the white mouse treated with $^{32}$P phenthoate at two dosages and Table 25 gives analogous data for the $^{14}$C label. $^{32}$P-labeled metabolites identified in varying amounts in either urine or feces were: demethyl phenthoate (LVII), demethyl phenthoate oxon (LVIII), phenthoate acid (LXI), O,O-dimethyl phosphorodithioic (LXII) and phosphorothioic (LXIII) acids. Unchanged phenthoate was present only in the feces. No phenthoate oxon (LVI) was detected in either urine or feces, despite the presence of other metabolites containing the P=O linkage such as LVIII and LXI. Of the various unknowns, D and E were common to both $^{32}$P and $^{14}$C labels. Based on its chromatographic behavior, which was similar to that of phenthoate acid (LIX) in the different TLC systems employed, the most likely candidate for the structure of E is O,O-dimethyl S-[$\alpha$-(carboxy)phenyl] phosphorothioate. However, this could not be confirmed since synthesis of this compound was not achieved. The other unknowns were highly polar substances which remained close to the origin of tlc plates developed with nonpolar solvents.

Metabolites LVII-LXI also were obtained from the white mouse treated with phenyl ring $^{14}$C phenthoate (Table 25) along with metabolites produced after removal of the dimethoxyphosphinothioyl moiety (LXIV-LXVII). A trace amount of phenthoate oxon (LVI) was observed in feces. Although noticeable differences in the relative amounts of several of the metabolites were observed between the $^{14}$C and $^{32}$P labels, on an overall basis the pattern of metabolism for the two labels was similar. It should be pointed out that direct comparisons between the two labels should not be made since the level of treatment at the two lower doses was different (17 and 30 mg/kg) and data presented for the aqueous phase for the $^{14}$C label are from analyses conducted 12 hours after treatment instead of the customary 24 hours. In this regard, numerous attempts to analyze the aqueous phase of the 24-hour $^{14}$C urine sample proved unsatisfactory owing to poor separation on tlc plates. Attempts to remove interfering materials by column chromatography, including the use of Sephadex G-50, or preparative tlc proved fruitless, and even in these cases undefined smears were obtained on analytical tlc plates. Mild acid treatment of the aqueous phase also had little effect in improving tlc separation. For reasons which were not obvious, this difficulty was not experienced with the urine samples containing $^{32}$P label nor with the 12-hour urine sample.

Examination of the data in Tables 24 and 25 reveals that the most prominent reactions leading to metabolic degradation of phenthoate in the mouse involves hydrolysis of the carboethoxy moiety or cleavage of the P-O (or possibly O-C), P-S, and C-S bonds. The metabolites produced from these reactions probably represent detoxication products. Of key significance is the virtual absence of phenthoate oxon (LVI), the metabolite which presumably is responsible for intoxication. Owing to

Table 24. METABOLITES ISOLATED FROM THE WHITE MOUSE 24 HOURS AFTER TREATMENT WITH $^{32}$P PHENTHOATE AT 30 AND 161 MG/KG

| | | % of Total recovered radioactivity | | | |
|---|---|---|---|---|---|
| | | Urine | | | |
| Metabolite | Dose | Aqueous phase | Organo-soluble | Feces | Total |
| LV | 30 | - | - | 2.6 | 2.6 |
| | 161 | - | - | 1.9 | 1.9 |
| LVII | 30 | 4.1 | - | - | 4.1 |
| | 161 | 7.1 | 0.1 | - | 7.2 |
| LVIII | 30 | 4.9 | 4.5 | - | 9.4 |
| | 161 | 8.3 | - | 0.4 | 8.7 |
| LIX | 30 | 0.3 | 3.9 | 0.5 | 4.7 |
| | 161 | - | 11.7 | 0.4 | 12.1 |
| LX | 30 | 24.2 | 1.6 | - | 25.8 |
| | 161 | 9.4 | 0.2 | 0.2 | 9.8 |
| LXI | 30 | 16.9 | 1.5 | - | 18.4 |
| | 161 | 4.6 | 0.2 | 0.4 | 5.2 |
| LXII | 30 | 15.3 | 0.5 | 1.1 | 16.9 |
| | 161 | 7.9 | 0.1 | 3.4 | 11.4 |
| LXIII | 30 | 7.7 | 0.3 | 1.2 | 9.2 |
| | 161 | 24.4 | - | 1.6 | 26.0 |
| Unknown A | 30 | 2.9 | - | 0.4 | 3.3 |
| | 161 | 1.3 | - | 0.4 | 1.7 |
| Unknown B | 30 | - | 2.9 | - | 2.9 |
| | 161 | 0.2 | - | 0.6 | 0.8 |
| Unknown C | 30 | - | 2.7 | - | 2.7 |
| | 161 | 1.8 | - | 0.4 | 2.2 |
| Unknown D | 30 | - | - | - | - |
| | 161 | 6.8 | 0.4 | - | 7.2 |
| Unknown E | 30 | - | - | - | - |
| | 161 | 4.1 | 1.4 | - | 5.5 |
| Unknown F | 30 | - | - | - | - |
| | 161 | 0.3 | - | - | 0.3 |
| Total | 30 | 76.3 | 17.9 | 5.8 | 100 |
| | 161 | 76.2 | 14.1 | 9.7 | 100 |

Table 25. METABOLITES ISOLATED FROM THE WHITE MOUSE 12 AND 24 HOURS AFTER TREATMENT WITH $^{14}$C PHENTHOATE AT 17 AND 161 MG/KG

| | | | % of Total Recovered Radioactivity | | | |
|---|---|---|---|---|---|---|
| | | | Urine | | | |
| | Metabolite | Dose | Aqueous phase[a] | Organo-soluble | Feces | Total |
| LV | Phenthoate | 17 | - | - | 3.2 | 3.2 |
| | | 161 | - | - | 5.1 | 5.1 |
| LVI | Phenthoate oxon | 17 | - | - | 0.3 | 0.3 |
| | | 161 | - | - | - | - |
| LVII | Demethyl phenthoate | 17 | - | - | 0.6 | 0.6 |
| | | 161 | 8.5 | 12.2 | 0.5 | 21.2 |
| LVIII | Demethyl phenthoate oxon | 17 | 28.3 | 6.7 | 0.1 | 35.1 |
| | | 161 | 9.6 | 1.7 | 0.1 | 11.4 |
| LIX | Phenthoate acid | 17 | 4.9 | 1.6 | 0.8 | 7.3 |
| | | 161 | 5.8 | 1.4 | 4.6 | 11.8 |
| LX | Demethyl phenthoate acid | 17 | 5.7 | 1.1 | 0.2 | 7.0 |
| | | 161 | 3.9 | - | - | 3.9 |
| LXI | Demethyl phenthoate oxon acid | 17 | 9.3 | 1.4 | - | 10.7 |
| | | 161 | 2.3 | - | - | 2.3 |
| LXIV | $[-SCH(C_6H_5)COOH]_2$ | 17 | 4.7 | 1.9 | 1.3 | 7.9 |
| | | 161 | 1.6 | 5.0 | 2.0 | 8.6 |
| LXV | $[-SCH(C_6H_5)COOEt]_2$ | 17 | - | - | 0.2 | 0.2 |
| | | 161 | - | - | 0.2 | 0.2 |
| LXVI | Mandelic acid | 17 | 2.6 | 1.4 | 0.7 | 4.7 |
| | | 161 | 4.8 | 2.6 | 1.1 | 8.5 |
| LXVII | $CH_3SCH(C_6H_5)COOH$ | 17 | - | 0.7 | - | 0.7 |
| | | 161 | 7.8 | - | - | 7.8 |
| | Unknown D | 17 | 4.4 | - | - | 4.4 |
| | | 161 | - | - | - | - |
| | Unknown E | 17 | - | 0.8 | - | 0.8 |
| | | 161 | - | - | 0.8 | 0.8 |
| | Total | 17 | 59.9 | 15.6 | 7.4 | 82.9 |
| | | 161 | 44.3 | 22.9 | 14.4 | 81.6 |

[a] Data for aqueous phase is for 12-hr urine sample.

the amounts of LVIII and LXI detected, desulfuration undoubtedly takes place, but it is not clear whether the thiono sulfur is replaced by oxygen before or after O-demethylation or hydrolysis of the carboethoxy moiety. If phenthoate oxon is produced by direct oxidative desulfuration of phenthoate, evidently it is rapidly metabolized further to LVIII and LXI and does not reach a critical level in the mouse.

Metabolism in House Flies - Data for the distribution and recovery of $^{32}P$ or $^{14}C$ phenthoate 4 hours after topical application to susceptible ($S_{NAIDM}$) and dimethoate resistant ($R_d$) strains of house flies are presented in Table 26. Treatment was at 5.0 and 50 µg/g, the respective $LD_{50}$ values for the $S_{NAIDM}$ and $R_d$ strains. At the time of treatment the $LD_{50}$ of dimethoate to the $R_d$ strain was approximately 150 µg/g. Based on recoveries in the internal fraction of flies treated at the lower dose, it is apparent that phenthoate is absorbed by the two strains at approximately equal rates. Thus, it appears that penetration is not a significant factor contributing to the 10-fold greater tolerance exhibited by the $R_d$ strain to phenthoate.

Results of the metabolism of $^{14}C$ phenthoate in the two strains are presented in Table 27. Because of the low specific activity of the $^{32}P$ label at the time of analysis, data from this label were less precise than those of the $^{14}C$ label and discussion will be restricted to data from the latter.

Qualitatively, the metabolism of phenthoate in house flies was similar to that in the white mouse. While slightly fewer metabolites were isolated from house flies, with the exception of the unknowns, those which were isolated from flies also were found in mice. The same metabolic reactions leading to detoxication products such as carboethoxy ester hydrolysis and cleavage of the P-O, P-S, and C-S bonds evidently also take place in house flies . Quantitatively, perhaps the most significant difference between mice and flies was the substantial amounts of phenthoate oxon detected in the latter. This metabolite, present at sufficiently high levels, readily accounted for the toxicity of phenthoate to house flies. Phenthoate also appeared to be relatively more stable in flies than in the mouse but direct comparisons were not possible from the data available owing to differences in the time and type of analyses. Total recovery of phenthoate, which includes internal extract, cage and external rinse, ranged from 35-67% of the applied dose 4 hours after treatment.

Owing to variability in the data, distinct differences in phenthoate metabolism between $S_{NAIDM}$ and $R_d$ house flies were not readily discernible. From the $^{14}C$ data (Table 27) penetration of phenthoate appeared to be slightly slower in the $R_d$ strain, but it is doubtful that this difference is significant. Of possible significance was the recovery of 2-fold more phenthoate oxon from $S_{NAIDM}$ (0.31 µg/g) than from $R_d$ (0.16 µg/g) flies treated at the same dose of 5 µg/g. While this difference is not large, it is in the proper direction. At the higher dose of 50 µg/g

Table 26. DISTRIBUTION OF RADIOACTIVITY 4 HOURS AFTER TOPICAL TREATMENT OF SUSCEPTIBLE ($S_{NAIDM}$) AND DIMETHOATE-RESISTANT ($R_d$) HOUSE FLIES WITH $^{14}C$- AND $^{32}P$-LABELED PHENTHOATE

| Label | Fly strain | Dose (μg/g) | % of Applied radioactivity recovered | | | |
|---|---|---|---|---|---|---|
| | | | Cage rinse | External rinse | Internal extract | Total |
| $^{32}P$ | $S_{NAIDM}$ | 5.0 | 24.1 | 29.1 | 28.3 | 81.5 |
| $^{14}C$ | $S_{NAIDM}$ | 5.0 | 11.2 | 26.7 | 54.2 | 92.1 |
| $^{32}P$ | $R_d$ | 5.0 | 21.9 | 29.4 | 34.2 | 85.5 |
| $^{14}C$ | $R_d$ | 5.0 | 12.3 | 40.1 | 42.8 | 95.2 |
| $^{32}P$ | $R_d$ | 50.0 | 16.8 | 45.9 | 28.2 | 90.9 |
| $^{14}C$ | $R_d$ | 50.0 | 17.8 | 55.2 | 24.1 | 97.1 |

Table 27. METABOLIC PRODUCTS RECOVERED FROM HOUSE FLIES AFTER TOPICAL TREATMENT WITH $^{14}C$ PHENTHOATE

| | % of Total recovered radioactivity[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $S_{NAIDM}$ (5.0 μg/g) | | | $R_d$ (5.0 μg/g) | | | $R_d$ (50 μg/g) | | |
| Metabolite | Cage rinse | External rinse | Internal rinse | Cage rinse | External rinse | Internal rinse | Cage rinse | External rinse | Internal rinse |
| LV | 4.1<br>(0.19) | 8.3<br>(0.38) | 23.2<br>(1.07) | 1.9<br>(0.09) | 24.3<br>(1.16) | 26.6<br>(1.27) | 10.5<br>(5.10) | 50.9<br>(24.71) | 5.9<br>(2.86) |
| LVI | 1.8<br>(0.08 | 1.8<br>(0.08) | 3.3<br>(0.15) | - | 0.9<br>(0.04) | 2.5<br>(0.12) | 0.3<br>(0.15) | - | 1.3<br>(0.63) |
| LVII | 1.2<br>(0.05) | 1.6<br>(0.07) | 9.2<br>(0.42) | 5.5<br>(0.26) | 1.8<br>(0.09) | 2.2<br>(0.11) | 1.8<br>(0.87) | 1.5<br>(0.73) | 6.7<br>(3.25) |
| LVIII | - | 5.8<br>(0.26) | 5.9<br>(0.27) | 5.5<br>(0.26) | 5.6<br>(0.27) | 3.3<br>(0.16) | 4.3<br>(2.09) | 1.5<br>(0.73) | 4.1<br>(1.99) |
| LIX | 1.8<br>(0.08) | 1.2<br>(0.05) | 3.3<br>(0.15) | - | 2.2<br>(0.11) | 2.2<br>(0.11) | - | - | 1.4<br>(0.68) |
| LX | - | 6.3<br>(0.29) | 3.9<br>(0.18) | - | - | 1.2<br>(0.06) | - | 0.2<br>(0.10) | - |
| LXI | - | - | - | - | - | 0.6<br>(0.03) | 0.8<br>(0.39) | 0.5<br>(0.24) | 1.0<br>(0.49) |
| LXIV | 2.0<br>(0.09) | 1.8<br>(0.08) | 2.9<br>(0.13) | - | 5.0<br>(0.24) | 3.5<br>(0.17) | 0.3<br>(0.15) | 0.3<br>(0.15) | 0.9<br>(0.44) |
| LXVI | 1.3<br>(0.06) | 0.6<br>(0.03) | 4.9<br>(0.23) | - | 2.2<br>(0.11) | 3.1<br>(0.15) | 0.3<br>(0.15) | 0.3<br>(0.15) | 0.5<br>(0.24) |

Table 27. continued

| Metabolite | Cage rinse | External rinse | Internal rinse | Cage rinse | External rinse | Internal rinse | Cage rinse | External rinse | Internal rinse |
|---|---|---|---|---|---|---|---|---|---|
| Unknown G | - | 1.6<br>(0.07) | 2.2<br>(0.10) | - | - | - | - | 1.7<br>(0.83) | 3.0<br>(1.46) |
| Total | 12.2 | 29.0 | 58.8 | 12.9 | 42.0 | 45.2 | 18.3 | 56.9 | 24.8 |

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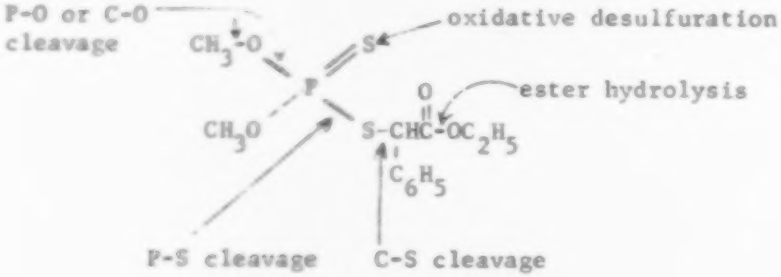
[a] Parenthetical values represent actual amounts (μg) of metabolites in terms of phenthoate equivalents.

($R_d$ strain $LD_{50}$), approximately 2.5-fold more $^{14}C$ oxon was recovered from the $R_d$ strain (0.78 µg/g) compared to the $S_{NAIDM}$) strain treated at 5.0 µg/g.

Conclusion - On the basis of the metabolic products identified, the metabolism of phenthoate in the white mouse and house fly appears to be similar to that which occurs in plants (see Part C-3). Further, in gross aspects the various metabolic reactions which phenthoate undergoes in these test animals correspond to those reported previously for malathion[11], a compound of similar structure and properties of selective toxicity. Indication of the functional groups in the phenthoate molecule which are metabolically labile is given in the structure below.



Metabolites generated from each or different combinations of these various reactions were identified. Secondary reactions involving the metabolic fragments, e.g., oxidative coupling to produce bis-[α-(carboethoxy)benzyl] disulfide (LXV) and bis-[α-(carboxy)benzyl] disulfide (LXIV), also occurred.

From the relative amounts of metabolites isolated from the mouse, no single reaction appeared to predominate over any of the others in the degradation of phenthoate. It is clear that the mouse is able to degrade phenthoate with almost equal facility by hydrolysis of the carboethoxy moiety, cleavage of the P-S or C-S bond, and removal of the methoxy group by either direct demethylation or hydrolytic cleavage of the P-O bond. As indicated earlier, little, if any, phenthoate oxon was detected, a finding which is in agreement with the low mammalian toxicity of phenthoate.

Comparison of the data for mouse metabolism with those of house flies reveals that hydrolysis of the carboethoxy moiety takes place to a greater extent in the mouse than in flies (compare metabolites LIX, LX, and LXI). This is in accord with earlier reports [12,13] where it was pointed out that carboxyesterase activity is substantially lower in insects than in mammals. On the other hand, degradation by loss of one of the methoxy groups appeared to be of major significance in flies.

Although a reasonable explanation for the selective toxicity of phenthoate may be based on differences in the amounts of phenthoate oxon recovered between the white mouse and house fly, the greater tolerance of the $R_d$ strain compared to the $S_{NAIDM}$ strain to phenthoate intoxication is less readily explainable owing to the relatively large amounts of oxon recovered from $R_d$ flies at the higher dosage. This phenomenon has been observed before in studies with other organophosphorus insecticides[14,15] and may be attributed to compartmentalization of the oxon at a locale distant from the site of action or possibly to decreased sensitivity of fly cholinesterase to inhibition[16].

Overall, the evidence indicates that phenthoate is metabolized through similar pathways in mice and house flies but with significant quantitative differences to account for its selective action.

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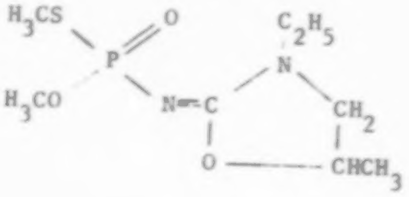
## C. STUDIES ON THE METABOLISM OF INSECTICIDES

In Part B of this report a number of studies on the comparative metabolism of selectively toxic insecticides in mammals and insects are described. These studies were conducted for the elucidation of the fundamental mechanisms responsible for insecticide selectivity. In addition to metabolism studies of this nature, work also was carried out for toxicological purposes and the assessment of hazards arising from the use of insecticidal chemicals under current development. This part of the report is concerned with these studies.

### 1. Metabolism of 2-[Methoxy(methylthio)phosphinylimino]-3-Ethyl-5-Methyl-1,3-Oxazolidine in the Cotton Plant and House Flies

Introduction - 2-[Methoxy(methylthio)phosphinylimino]-3-ethyl-5-methyl-1,3-oxazolidine (I) or Stauffer R-16,661, is a new experimental insecticide which in our screening tests has demonstrated outstanding systemic activity in cotton plants against a wide variety of insects. The structure of I is given below.



In greenhouse screening tests this material was equal, or superior, to aldicarb as a systemic insecticide, following granular treatment of the soil or topical application on the stem, against such typical cotton pests as the red spider mite (*Tetranychus cinnabarinus*), cotton aphid (*Aphis gossypii*), and cotton leaf perforator (*Bucculatrix thurberiella*). I also was effective against the pink bollworm (*Pectinophora gossypiella*), giving 100% control for at least 40 days after treatment.

The principal drawback which prevents the use of this potent systemic material in practical field application is its extremely high mammalian toxicity. In our tests the $LD_{50}$ of I to the white mouse was 0.1-0.2 mg/kg, a value which makes it approximately 10-fold more toxic to the mouse than aldicarb. However, because of its outstanding systemic properties, it was decided to study the metabolism of this material in plants and insects in an effort to determine whether the compound itself or whether metabolic products were responsible for insecticidal activity. Also, it was important to assess the toxicological properties of the metabolites, owing to the possibility that the metabolite might be less toxic to mammals but still retain toxicity to insects.

**Metabolism in the Cotton Plant** - $^{32}$P-labeled I, prepared through a 2-step synthesis beginning with $^{32}$P O,O-dimethyl phosphorochloridothioate, was used for this study. For the study of the metabolism of I in the cotton plant, two different methods of treatment of the plant were used. In the first method, the base of a young cotton plant containing developing bolls was treated with a total of 25 $\mu$l (37.5 mg) of $^{32}$P-I. The plant was maintained in the greenhouse and leaves were taken from the upper and lower sections of the plant for periodic assay. In the second method, ten freshly cut cotton petioles were placed in 10 ml water containing 10 mg $^{32}$P-I and allowed to stand overnight. The aqueous solution containing I was completely absorbed after 24 hr and the petioles were removed to fresh water and kept in the greenhouse. At various time intervals, petioles were removed and examined for metabolism.

I is very stable in the cotton plant. For example, 14 days after stem application 86-93% of the total radioactivity isolated from cotton leaves was in the form of unchanged I. After 51 days the major component was still I but quantitation was not possible owing to low radioactivity from decay. Similar results were obtained when isolated cotton petioles were treated, as shown in Table 1. The $R_f$ values reported in this table were determined on silica gel HF-254 (Merck, Germany) using 95% ethanol as the developing solvent. The data in the table show that I is the principal component in the leaf although several metabolites also are formed but in relatively small amounts. The metabolites at $R_f$ 0.0 and 0.05 are probably hydrolysis products since the compound 2-[hydroxy(methylthio)phosphinylimino]-3-ethyl-5-methyl-1,3-oxazolidine, the O-demethylated product isolated from the alkaline hydrolysis of I showed an $R_f$ of 0.1 on the same tlc system. However, it is possible also that these metabolites may be plant conjugates. The principal intact metabolite was found at $R_f$ 0.58 and this compound undoubtedly is the 4-keto derivative, II, formed by oxidation of the 4-position in the oxazolidine ring. Support for this structure will be presented later. Another metabolite which appeared in small but varying amounts was found at $R_f$ 0.33. The structure of this compound is tentatively suggested as the 4-OH derivative, III, the precursor to the 4-keto metabolite.

The major tlc component found at $R_f$ 0.48 was unequivocably established as unchanged I. This material was isolated from cotton leaves by means of column and repeated thin-layer chromatography. The PMR spectrum of the isolated material was virtually identical to that of I with a $^3$H triplet at $\delta$ 1.20 for the methyl protons on N-ethyl, a $^3$H doublet centered at $\delta$ 1.53 for the 5-methyl, a $^1$H multiplet at $\delta$ 4.80 for the proton on the 4-carbon atom, a multiplet near $\delta$ 3.2 for the pair of methylene protons adjacent to the ring nitrogen, a $^3$H doublet for S-$CH_3$ protons at $\delta$ 2.3 (J = 14 Hz), and a $^3$H doublet at $\delta$ 3.81 (J = 13 Hz) for O-$CH_3$ protons.

Table 1. AMOUNTS OF R-16,661 (I) AND METABOLITES FOUND IN COTTON PETIOLES AFTER VARYING TIME INTERVALS

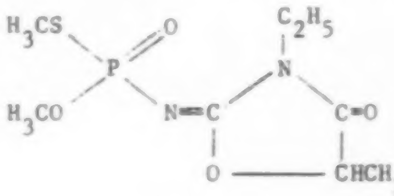
| Compound | $R_f$ | % of Recovered radioactivity after indicated days following treatment | | | |
|---|---|---|---|---|---|
| | | 7 | 14 | 21 | 28 |
| 4-Keto R-16,661 | 0.58 | 5.2 | 3.3 | 3.7 | 6.2 |
| R-16,661 (I) | 0.48 | 89 | 75 | 73 | 80 |
| 4-OH R-16,661 (tentative) (III) | 0.33 | trace | trace | trace | trace |
| Unknown | 0.05 | 2.4 | 8.0 | 7.5 | 7.2 |
| Unknown | 0 | 1.0 | 7.7 | 5.2 | 1.6 |

Metabolism in the House Fly and by In Vitro Enzyme Systems - In general, the metabolism of I in the house fly was similar to that occurring in the cotton plant. I penetrated very slowly into the fly and 20 hr after a 2 μg/♀ topical application only 12% and 7% of the applied dose was found internally in the susceptible $S_{NAIDM}$ and parathion resistant $R_p$ strains, respectively (Table 2). As in the case with the cotton plant, unchanged I was the major component found along with a significant amount of the 4-keto derivative (II). The unknown at $R_f$ 0.0 again probably represents hydrolyzed material or possibly conjugates. The metabolite suggested as the 4-OH derivative (III), however, was not detected in flies.

In vitro metabolism studies using house fly and mosquito larvae homogenate resulted in tlc patterns of metabolites similar to those obtained with living houseflies. In all these cases, the metabolite believed to be the 4-OH derivative was not detected. Mouse liver microsome-plus-soluble enzyme fraction, on the other hand, produced a metabolic pattern virtually identical to that found in the cotton plant. The results are indicated in Table 3.

Oxidation by the Udenfriend Model System - Incubation of I in the Udenfriend model oxidation system gave products with identical chromatographic behavior to those obtained from the cotton plant and mouse liver preparation. Comparison was made by use of two different tlc systems. Since earlier attempts to isolate the pure 4-keto derivative (II), ($R_f$ 0.48 using HF-254-95% ethanol; $R_f$ 0.8 using silica gel G-chloroform-ethanol 4:1) from cotton leaves treated with I proved unsuccessful owing to contamination by plant material, the Udenfriend system was turned to for the synthesis of this metabolite. A variety of other synthetic approaches for the preparation of II were tried earlier but failed.

After incubating 0.79 g of I in the Udenfriend system for a total of 72 hr, and after repeated thin-layer chromatography approximately 5 mg of a yellow oil was isolated without impurity which from spectroscopic evidence was proved to be the 4-keto derivative. This material was chromatographically identified by two tlc systems to the material isolated from the cotton plant. The mass spectrum of this compound was consistent with the 4-keto structure shown below. In addition to the parent ion (M = 266), the cleavage of $-CH_3$ (M-15), $-OCH_3$



(M-31) and $-SCH_3$ (M-47) radicals from the parent ion confirmed the presence of the O,S-dimethyl phosphorothioyl moiety. The cyclic amide

Table 2. DISTRIBUTION OF I IN SUSCEPTIBLE ($S_{NAIDM}$) AND PARATHION-RESISTANT ($R_P$) HOUSE FLIES 20 HR AFTER TREATMENT

| Fraction | Compound | $R_f$[a] | % of Recovered radioactivity $S_{NAIDM}$ | % of Recovered radioactivity $R_P$ |
|---|---|---|---|---|
| External | - | - | 89.1 | 92.7 |
| Internal | Unknown | 0.0 | 0.5 | 0.1 |
| | I | 0.5 | 8.9 | 7.0 |
| | 4-Keto (II) | 0.6 | 1.5 | 0.2 |

[a] tlc; silica gel G, developed first with benzene, then with 95% ethanol.

Table 3. METABOLISM OF I BY MOUSE LIVER MICROSOME-PLUS-SOLUBLE ENZYME PREPARATION

| Compound | $R_f$[a] | % of Recovered radioactivity |
|---|---|---|
| Unknown | 0.0 | 4.3 |
| 4-OH (tentative) (III) | 0.6 | 1.5 |
| I | 0.7 | 91.0 |
| 4-Keto (II) | 0.8 | 3.1 |

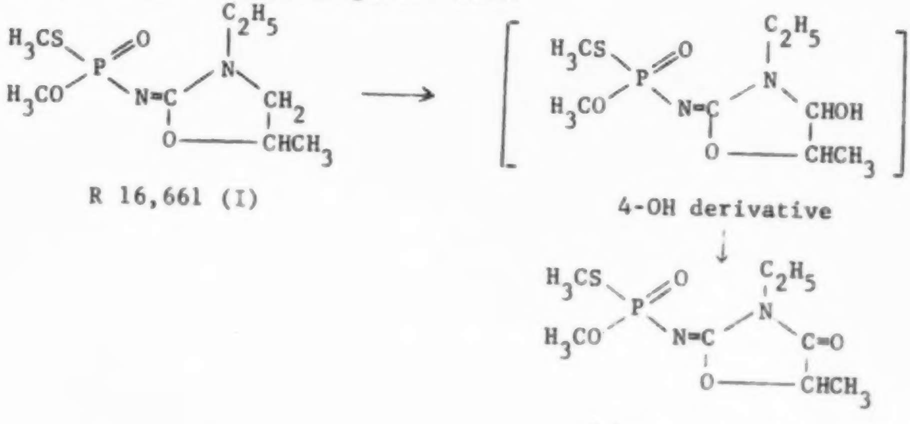
[a] tlc; silica gel G, developed first with 1:2 benzene-ethyl acetate, then with 4:1 ethanol-chloroform.

structure was confirmed by loss of ethylene ($C_2H_4$ = 28) and carbon monoxide (CO = 28), and the presence of a base peak (m/e = 43) for $CH_3CO+$. A PMR spectrum taken in d-chloroform (TMS) also confirmed the 4-keto structure.

Attempt also was made to isolate the less important metabolite $R_f$ 0.6 in Table 3 ($R_f$ 0.33 using HF-254-95% of ethanol system), but not enough material could be obtained free from impurity for definitive spectroscopic analysis. The assignment of a hydroxylated structure, probably 4-OH, to the metabolite is based primarily on tlc behavior relative to I and to its likelihood as a logical precursor to the 4-keto metabolite. In the various tlc systems used in this study, the rate of movement of each compound is inversely related to its polar property and, therefore, the 4-OH compound would be expected to have a lower $R_f$ value compared to I.

Toxicological Properties - Toxicological properties for I and II are presented in Table 4. The data show that compared to I, the 4-keto metabolite is approximately 10-fold less effective in inhibiting insect and mammalian cholinesterase and is 15-fold less toxic to the house fly. On the other hand, the two compounds were almost equally toxic to the white mouse. Thus, the 4-keto metabolite appears to be selectively toxic in the wrong direction, i.e., it is nontoxic to the house fly but highly toxic to the mouse.

Conclusion - R 16,661 (I), an outstanding systemic insecticide, evidently is quite stable in the cotton plant and in the house fly. It is, however, slowly metabolized in these biological systems to give as one of the eventual principal metabolites the 4-keto derivative, probably according to the metabolic scheme given below.



R 16,661 (I)

4-OH derivative

4-keto derivative (II)

Table 4. TOXICOLOGICAL PROPERTIES OF I AND ITS 4-KETO (II) METABOLITE

| | I | II |
|---|---|---|
| Anti-ChE ($I_{50}$ M)[a] | | |
| Fly-head ChE | $3.0 \times 10^{-8}$ | $2.7 \times 10^{-7}$ |
| Bovine erythrocyte ChE | $5.3 \times 10^{-7}$ | $5.6 \times 10^{-6}$ |
| Toxicity, $LD_{50}$ (mg/kg) | | |
| House fly | 16 | 240 |
| White mouse | 0.1-0.2 | 0.4-0.6 |

[a] $I_{50}$ value after 10-min incubation period.

Because of the stability of I in the cotton plant and the much lower insecticidal activity of the major metabolite, the 4-keto derivative (II), it appears that I itself is responsible for systemic activity. Based on our toxicological data the metabolic coversion of I to the 4-keto derivative in the plant is an undesirable occurrence owing to the relatively poor insecticidal activity and high mammalian toxicity of the metabolite.

The structure of I is unusual among the contemporary organophosphorus insecticides and compounds of this type deserve further study. For example, based on its structure it is not obvious which moiety will be displaced when this compound reacts to inhibit the cholinesterase enzyme. The fact that $CH_3O$-P bond cleavage occurs when I is treated with sodium hydroxide suggests that methoxide ion is the leaving group, even though thiomethylate ion is a more likely candidate based on reactivity grounds.

In many respects I is similar in structure and properties to the *O*-alkyl *S*-alkyl phosphoramidothioates, which have recently gained prominence as effective insecticides[2]. The mode of action of the phosphoramidothioates, at this stage, however, is not well understood.

### 2. Metabolism of O-(4-Bromo-2,5-Dichlorophenyl) O-Methyl Phenylphosphonothioate (Leptophos) in White Mice, on Cotton Plants, and in House Flies

**Introduction** - Leptophos [*O*-(4-bromo-2,5-dichlorophenyl) *O*-methyl phenylphosphonothioate] (IV) is a new phosphonate insecticide which has shown promise for the control of a broad spectrum of insect pests and as a fungicide against rice stem blast. However, a recent report[3] has implicated it as a material possessing delayed neurotoxic activity. Leptophos has been accused as the probable cause of paralysis and death of about 1,300 water buffalo in the Nile delta during the summer, 1971[4]. In order to develop information on the mode of action and metabolism of leptophos in animals and plants, this material was examined in the white mouse, cotton plant, and house fly.

**Metabolism in the White Mouse** - White mice were treated orally with two different labels of $^{14}$C leptophos, i.e., *O*-(4-bromo-2,5-dichlorophenyl-$^{14}$C) (phenoxy label), and phenyl-$^{14}$C-phosphonothioate (phenyl label). The results of metabolism studies performed in the white mouse treated with 25 mg/kg *phenoxy* and *phenyl* $^{14}$C-labeled leptophos are summarized in Tables 5, 6, and 7.

In the case of *phenoxy*-labeled leptophos, radioactivity was rapidly eliminated from the mouse and elimination was virtually complete 48 hours after treatment, the bulk of the excreted material being present in the urine (Table 5). In comparison, elimination of *phenyl*-labeled radioactivity was notably slower and radioactivity was detected in the urine as long as 144 hours following treatment. It was estimated that

Table 5. DISTRIBUTION OF LEPTOPHOS AND ITS METABOLITES AT DIFFERENT TIME INTERVALS AFTER ORAL TREATMENT OF A WHITE MOUSE AT 25 MG/KG

| Position of label | Time (hr) | % in urine | % in feces | Total[a] |
|---|---|---|---|---|
| phenoxy | 7 | 58.1 | 1.0 | 59.1 |
| " | 24 | 29.9 | 2.2 | 32.1 |
| " | 48 | 18.1 | 1.1 | 19.2 |
| | | Total recovery | | 110.3 |
| phenyl | 7 | 43.8 | 1.1 | 44.9 |
| " | 24 | 23.9 | 7.7 | 31.6 |
| " | 48 | 6.9 | trace[b] | 6.9 |
| " | 72 | 2.7 | trace[b] | 2.7 |
| " | 144 | 1.8 | 0 | 1.8 |
| | | Total recovery | | 87.9 |

[a] The total recovery shown was based on the amount administered. The error in administered dose is estimated to be 10%.

[b] "trace" refers to less than 0.1% radioactivity administered.

the error in the administered dose was approximately ± 10% and, therefore, that the total amounts recovered were close to complete recovery of radioactivity. The rate in which the two labels in leptophos are eliminated, in urine and feces, is presented graphically in Fig. 1. In spite of the possible error in administration of the radioactive compound, there is little doubt that the carbon-14 in the phenyl group is eliminated at a slower rate than that in the phenoxy group. A possible explanation for the difference in elimination rates is that the conjugate of the phenoxy-labeled phenol (IX) is eliminated more rapidly than the phenyl-labeled phenylphosphonic acids (VI, VII, and VIII).

The major portion of the radioactivity eliminated from the mouse was in the form of degraded products present primarily in the urine (Table 6 and Table 7). Leptophos and the oxon (V) were present in small amounts but essentially all in the feces. With *phenoxy*-labeled leptophos, more than 90% of the urinary metabolite was in the form of conjugated 4-bromo-2,5-dichlorophenol, although a small amount of free phenol also was present. The exact nature of the conjugate was not established but both enzymatic ($\beta$-glucuronidase/aryl sulfatase) and acid hydrolysis yielded only the phenol (IX).

The *phenyl*-labeled leptophos gave *O*-methyl phenylphosphonothioic acid (VI), *O*-methyl phenylphosphonic acid (VII), and phenylphosphonic acid (VIII) as the principal degradative products (Table 7) present entirely in the urine. Compound VI was the major component present in the early collection periods but its amount decreased rapidly with time, eventually reaching a level about equal to that of VIII. As in the *phenoxy* label, both leptophos and leptophos oxon were found in the feces, although unexplainably larger amounts of unchanged leptophos were detected with the *phenyl* label.

The unknown metabolite(s) listed in Table 6 and 7 cochromatographed with an authentic sample of *O*-(4-bromo-2,5-dichlorophenyl) phenylphosphonic acid on tlc using Silica HF-254 with a developing solvent of benzene-chloroform (1:1).

*Cotton Plant Metabolism* - Data for the metabolism of leptophos after application on the surface of cotton leaves at a dosage equivalent to one lb/acre are presented in Tables 8, 9, and 10. From the data in Table 8, it is evident that leptophos does not penetrate readily into the leaf but remains on the surface or is volatilized. The primary mechanism by which leptophos is lost appears to be by volatilization since there was a steady decrease in total measurable radioactivity with time. For example, 73-75% of the applied leptophos was recovered one week after treatment compared to 19-21% after five weeks. Relatively small amounts of radioactivity were found inside the leaf either as benzene or methanol extracts or bound to the pulp. It should be pointed out that control experiments were carried out to show that

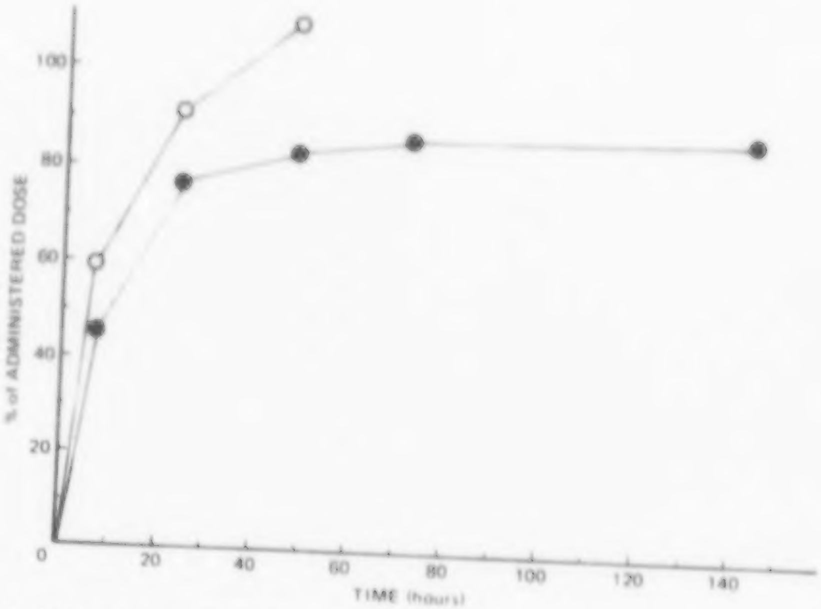
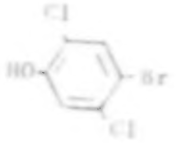
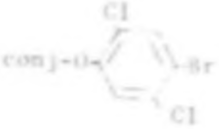


Fig. 1. Rate of elimination of radioactivity from the white mouse, in urine and feces, after oral administration of *phenoxy*-labeled ( ○ ) and *phenyl*-labeled ( ● ) leptophos

Table 6. METABOLITES FOUND AT VARIOUS TIMES[a] AFTER ORAL TREATMENT OF A WHITE MOUSE USING $^{14}C$-PHENOXY-LABELED LEPTOPHOS AT 25 MG/KG

| Compound | 7 hr | 24 hr | 48 hr | Total |
|---|---|---|---|---|
| IV Leptophos | 0.7 | 0.8 | 0.2 | 1.7 |
| V Leptophos oxon | 0.7 | 0.2 | 0 | 0.9 |
| IX  | 4.5 | 1.2 | 1.5 | 7.2 |
|  | 47.0 | 26.5 | 15.6 | 89.1 |
| Unknown[b] | 0.6 | 0.5 | trace[c] | 1.1 |

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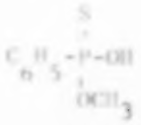
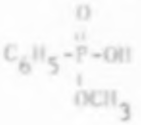
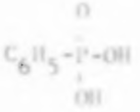
[a] Values are given as per cent of recovered $^{14}C$ material.

[b] The unknown material was not conclusively identified but it co-chromatographs with an authentic sample of O-(4-bromo-2,5-dichlorophenyl) phenylphosphonic acid.

[c] "trace" refers to less than 0.1% of recovered materials.

Table 7. METABOLITES FOUND AT VARIOUS TIMES[a] AFTER ORAL TREATMENT OF A WHITE MOUSE USING $^{14}$C-PHENYL-LABELED LEPTOPHOS AT 25 MG/KG

| Compound | | 7 hr | 24 hr | 48 hr | 72 hr | 144 hr | Total |
|---|---|---|---|---|---|---|---|
| IV | Leptophos | 1.2 | 8.2 | trace[b] | trace[b] | trace[b] | 9.4 |
| V | Leptophos oxon | 0 | 0.4 | trace[b] | trace[b] | trace[b] | 0.4 |
| VI |  $C_6H_5-\overset{S}{\overset{\Vert}{P}}-OH$ with $\underset{OCH_3}{\vert}$ | 35.4 | 15.0 | 4.6 | 1.0 | 0.6 | 56.6 |
| VII |  $C_6H_5-\overset{O}{\overset{\Vert}{P}}-OH$ with $\underset{OCH_3}{\vert}$ | 8.9 | 5.7 | 1.5 | 1.1 | 0.7 | 17.9 |
| VIII |  $C_6H_5-\overset{O}{\overset{\Vert}{P}}-OH$ with $\underset{OH}{\vert}$ | 5.5 | 6.3 | 1.8 | 1.1 | 0.8 | 15.5 |
| | Unknown[c] | 0 | 0.2 | 0 | 0 | 0 | 0.2 |

[a] Values are given as per cent of recovered $^{14}$C material.

[b] "trace" refers to less than 0.1% recovered material.

[c] The unknown material was not conclusively identified but it cochromatographs with an authentic sample of O-(4-bromo-2,5-dichlorophenyl) phenylphosphonic acid.

Table 8. DISTRIBUTION[a] OF LEPTOPHOS AND ITS METABOLITES AT DIFFERENT TIME INTERVALS AFTER TOPICAL APPLICATION TO COTTON PLANTS

| | 1 week | 3 week | 5 week | 9 week |
|---|---|---|---|---|
| Phenoxy label | | | | |
| Wash | 64.3 | 16.5 | 9.0 | 1.8 |
| Benzene extract | 5.6 | 6.2 | 4.5 | 2.4 |
| Methanol extract | 4.9 | 9.9 | 4.2 | 3.7 |
| Pulp (bound) | trace[b] | 1.8 | 2.9 | 3.1 |
| Total | 74.8 | 34.4 | 20.6 | 11.0 |
| Phenyl label | | | | |
| Wash | 62.1 | 18.9 | 7.8 | |
| Benzene extract | 7.0 | 8.1 | 4.8 | |
| Methanol extract | 4.3 | 10.0 | 3.7 | |
| Pulp (bound) | trace[b] | 1.1 | 2.2 | |
| Total | 73.4 | 38.1 | 18.5 | |

---

[a] Values are given in per cent based on amount of radioactive compound applied.

[b] "trace" refers to less than 0.1%.

Table 9. METABOLITES[a] FOUND AT VARIOUS TIMES AFTER TOPICAL APPLICATION OF $^{14}$C PHENOXY-LABELED-LEPTOPHOS TO COTTON PLANTS

| | 1 week | 3 week | 5 week | 9 week |
|---|---|---|---|---|
| Leptophos (IV) | | | | |
| Surface wash | 80.7 | 37.8 | 35.1 | 29.3 |
| Solvent extract | 10.7 | 23.6 | 22.8 | 13.6 |
| Pulp | 0 | 0 | 0 | 0 |
| Leptophos oxon (V) | | | | |
| Surface wash | 0 | trace | 0 | 0 |
| Solvent extract | 0 | 0 | trace | 0 |
| Pulp | 0 | 0 | 0 | 0 |
| Salt of phenol (IX)[b] | | | | |
| Surface wash | 5.3 | 9.9 | 8.7 | 7.2 |
| Solvent extract | 3.3 | 23.2 | 19.4 | 21.8 |
| Pulp | 0 | 0 | 0 | 0 |
| Unknown[c] | | | | |
| Surface wash | 0 | trace | 0 | 0 |
| Solvent extract | 0 | 0.3 | 0 | 0 |
| Pulp | 0 | 5.2 | 14.0 | 28.1 |

[a] Values are given as per cent based on the total amount of radioactivity recovered.

[b] The values reported are a sum of the free phenol (IX) and a salt of phenol (IX).

[c] These values reflect the amount of unextractable radioactive material bound in the pulp.

Table 10. METABOLITES[a] FOUND AT VARIOUS TIMES AFTER TOPICAL APPLICATION OF $^{14}$C-PHENYL LABELED LEPTOPHOS TO COTTON PLANTS

| | 1 week | 3 week | 5 week | | 1 week | 3 week | 5 week |
|---|---|---|---|---|---|---|---|
| Leptophos (IV) | | | | Acid (VII) | | | |
| Surface wash | 82.1 | 48.2 | 37.5 | Surface wash | 0.8 | 0.3 | 0.5 |
| Solvent extract | 14.3 | 30.1 | 31.3 | Solvent extract | 0.5 | 0.3 | 1.6 |
| Pulp | 0 | 0 | 0 | Pulp | 0 | 0 | 0 |
| Leptophos oxon (V) | | | | Acid (VIII) | | | |
| Surface wash | 0 | 0 | 0 | Surface wash | 0 | 0 | 0 |
| Solvent extract | 0 | 0 | 0 | Solvent extract | trace | 16.2 | 10.3 |
| Pulp | 0 | 0 | 0 | Pulp | 0 | 0 | 0 |
| Acid (VI) | | | | Unknown[b] | | | |
| Surface wash | 1.6 | 1.3 | 4.3 | Surface wash | 0 | 0 | 0 |
| Solvent extract | 0.7 | 0.8 | 2.7 | Solvent extract | 0 | 0 | 0 |
| Pulp | 0 | 0 | 0 | Pulp | trace | 2.8 | 11.8 |

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[a] Values are given as per cent based on the total amount of radioactive compound recovered.

[b] These values reflect the amount of unextractable radioactive material bound in the pulp.

radioactivity was not being lost in the work-up procedure, i.e., greater than 90% of the applied radioactivity was recovered.

Qualitatively, the degradation of leptophos in or on the cotton leaf is similar to that occurring in the white mouse (cf Tables 9 and 10). The major component present in the recoverable radioactivity one week after application was unchanged leptophos (91-96%) and this substance gradually diminished with time to approximately 29% in 9 weeks. Most of the leptophos remained on the leaf surface although significant but small amounts evidently were absorbed into the leaf. Little, if any, leptophos oxon (V) was present at any sampling period although other alteration products, i.e., the phenol (IX), in the form of an unknown salt, and the phenylphosphonic acid derivatives VI, VII, and VIII were present in varying amounts. No free or conjugated phenol was detected and essentially all of the phenol was isolated as a salt (Table 9). This substance was the principal degradation product present in the leaf but substantial amounts also were found on the leaf surface. The unknown material(s) from the phenoxy label treatment was present almost entirely in the pulp and, because it was not extractable with methanol, it was considered bound to the parent plant pulp.

Results obtained with the phenyl labeled leptophos show that all three phenylphosphonic acid derivatives (VI, VII, VIII) were present both in and on the cotton leaf. The largest component among the acids was phenylphosphonic acid, particularly at the later sampling periods. As in the case of the phenoxy label, a significant amount of the measurable radioactivity was inseparable from the pulp by methanol extraction and also was considered to be bound to the pulp. The nature of this bound material, of course, is unknown but it undoubtedly is not the same as the bound material obtained from plants treated with the phenoxy-labeled leptophos.

Metabolism in House Flies - Two different strains of house flies were used in this study, a susceptible ($S_{NAIDM}$) and parathion resistant ($R_P$) strain. The toxicity of leptophos to the $S_{NAIDM}$ strain was 11 μg/g and >500 μg to the $R_P$ strain.

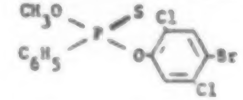
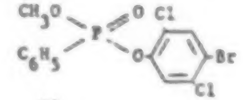
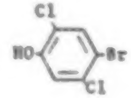
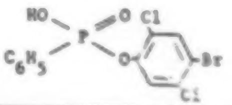
The penetration of different dosages of leptophos into susceptible and resistant flies was determined 1, 2, 4, and 6 hours after application using $^{14}$C-phenoxy leptophos. Penetration rate, as estimated from the amount of radioactivity present in the "external recovery" and "internal recovery," was remarkably slow in both strains of flies. For example, at a dosage of 10 μg/g the disappearance of radioactivity from the external surface of the $R_P$ strain was less than 5% after 1 hour. After 4 hours, 64.7% and 88.8% of the applied radioactivity were recovered from the external surface of $S_{NAIDM}$ and $R_P$ flies, respectively, and, correspondingly, 30% and 10% were recovered in the internal fraction.

Proportionately lesser amounts of leptophos penetrated into house flies treated at 500 μg/g. This is shown graphically in Fig. 2 where comparison is made between the rate of penetration of leptophos into house flies treated at 10 and 500 μg/g. The figure clearly reveals a marked difference in the pentration rate of leptophos into the two strains of house flies. Penetration into the resistant $R_{parathion}$ strain was abnormally slow and even after 6 hours approximately 85 and 95% of the applied dosages of 10 and 500 μg/g were recovered from the external surface of susceptible and resistant flies, respectively. The unusually low rate of penetration of leptophos into the $R_P$ strain suggests that reduced penetration is probably an important contributing factor to resistance in this strain. Further, the generally poor penetrating ability of leptophos into both strains is consistent with the observed delay in onset of toxic symptoms and the relatively poor toxicity of leptophos to house flies compared to parathion.

Results indicating the nature and quantity of the various metabolites recovered from house flies 4 hours after treatment with $^{14}$C-phenoxy leptophos are presented in Table 11. Data are given for susceptible ($S_{NAIDM}$) flies treated at 10 μg/g and resistant ($R_P$) flies treated at 10 ($R_1$) and 500 ($R_2$) μg/g. A brief explanation of the data under the heading "Fly homogenate, water soluble" is appropriate since the results under this column may be misunderstood. In the experimental procedure for the extraction of metabolites present internally in the fly, the methanol-acetone extract was concentrated to dryness, the residual material was suspended in distilled water, and the mixture was extracted twice with a benzene-hexane-chloroform mixture. This extract was labeled the "organosoluble" fraction. The remaining aqueous phase was acidified, placed in a boiling water bath for 15 minutes to hydrolyze conjugates, cooled and extracted with the same organic solvent mixture. The material in this extract was labeled "water soluble." Brief reiteration of the experimental procedure is made here because of the substantial amounts of leptophos and leptophos-oxon which were recovered after acid treatment. Since it is not reasonable for these compounds to form conjugates, their retention in the aqueous phase after extraction with organic solvent prior to acid treatment is not readily accountable. Evidently, leptophos and leptophos-oxon are tightly bound in some manner to house fly tissue and treatment with dilute acid is required to release them for extraction by organic solvents.

Except for significant differences in the penetration rate, large quantitative differences in the metabolism of leptophos between susceptible and resistant house flies were not observed. At the lower dosage of 10 μg/g almost 6-fold more radioactivity was detected internally in susceptible than in resistant house flies. The data in Table 11 show that leptophos is quite stable in both strains of flies and a major portion of the radioactivity found internally in the flies was the parent material. The data reveal that slightly more leptophos-oxon was present in the internal fly homogenate of susceptible

Table 11. Metabolic Products Recovered from $S_{NAIDM}$ and $R_{parathion}$ Houseflies 4 Hours After Topical Application of $^{14}$C-Phenoxy Leptophos

| | | Per cent total applied radioactivity[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Internal fly homogenate | | | | | |
| | $R_f$ Benzene-chloroform | Flask rinse | | | Fly rinse | | | Organosoluble | | | Water soluble | | |
| Metabolite | (1:1) | $S_{NAIDM}$ | $R_1$[b] | $R_2$[c] | $S_{NAIDM}$ | $R_1$[b] | $R_2$[c] | $S_{NAIDM}$ | $R_1$[b] | $R_2$[c] | $S_{NAIDM}$ | $R_1$[b] | $R_2$[c] |
|  | 0.65 | 27.9<br>(2.79) | 5.53<br>(0.55) | 4.70<br>(23.50) | 45.91<br>(4.59) | 60.48<br>(6.05) | 75.48<br>(377.4) | 10.55<br>(1.06) | - | 4.00<br>(20.00) | 21.80<br>(2.18) | 5.23<br>(0.52) | 4.35<br>(21.75) |
|  | 0.25 | 1.33<br>(0.13) | 0.56<br>(0.06) | - | 1.80<br>(0.18) | 2.53<br>(0.25) | 1.35<br>(6.75) | 0.42<br>(0.04) | - | 0.24<br>(1.20) | 1.60<br>(0.16) | 0.46<br>(0.05) | 1.53<br>(7.65) |
|  | 0.39 | - | 2.51<br>(0.25) | - | 0.46<br>(0.05) | 14.61<br>(1.46) | 1.72<br>(8.60) | 0.30<br>(0.03) | - | 1.03<br>(5.15) | 4.08<br>(0.41) | 0.26<br>(0.03 | 1.00<br>(5.00) |
|  | 0.13 | 0.66<br>(0.07) | 0.23<br>(0.02) | 0.42<br>(2.10) | 2.33<br>(0.23) | 1.34<br>(0.13) | 2.15<br>(10.75) | 0.41<br>(0.04) | - | 0.03<br>(0.15) | 0.08<br>(0.01) | 0.05<br>(0.01) | 1.08<br>(5.40) |
| | Total[d] | 4.78 | 8.77 | 5.12 | 50.50 | 78.96 | 80.70 | 11.68 | ~1.0 | 5.3 | 27.56 | 6.0 | 7.96 |

---

[a] Parenthetical values represent amounts in µg/g

[b] $R_1 = R_{parathion}$ (10 µg/g)

[c] $R_2 = R_{parathion}$ (500 µg/g)

[d] % Recovery, $S_{NAIDM}$ = 94.5%, $R_1$ = 94.7%, $R_2$ = 99.1%

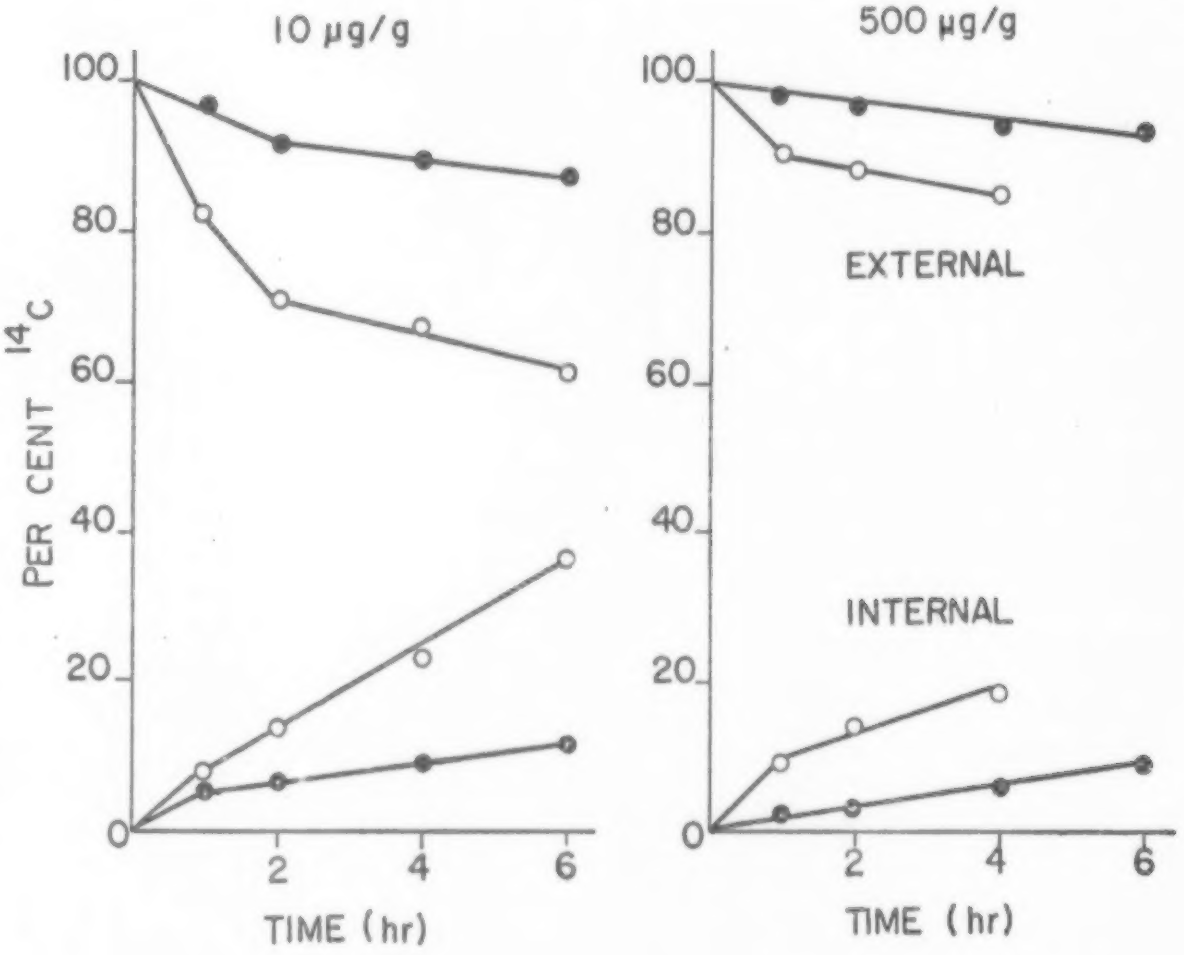


Figure 2. RATE OF PENETRATION OF LEPTOPHOS AT LOW AND HIGH DOSAGE INTO $S_{NAIDM}$ (○) and $R_p$ (●) HOUSE FLIES

flies, but a larger amount of the oxon was present in the external rinse of the resistant flies. The most striking difference between the two strains, however, was the relatively large quantity of the phenol in the external rinse of resistant flies. In general, surprisingly large amounts of metabolites were found in the external rinse and these materials probably were readsorbed on to the flies through physical contact with fly excreta.

$R_P$ flies treated with leptophos at 500 µg/g developed approximately the same symptoms of intoxication as $S_{NAIDM}$ flies treated at 10 µg/g. At this higher dosage, the percentage of applied leptophos penetrating into resistant flies was less than that for the susceptible strain at the lower dosage but owing to the 50-fold more leptophos applied, the actual quantity of penetrated material was substantially greater. This is particularly significant with respect to leptophos-oxon since this metabolite is undoubtedly the agent responsible for cholinesterase inhibition and intoxication. For example, the actual amount of the oxon detected in the homogenate of resistant flies treated with 500 µg/g was 0.177 µg/fly while the amount detected in susceptible flies treated with 10 µg/g was 0.004 µg/fly, a difference of approximately 40 fold. Since the sensitivity of the cholinesterases from the two strains of flies to inhibition by leptophos-oxon is virtually identical, the large difference in oxon levels in the two strains is in strong contrast to the toxic symptoms observed.

Concurrent studies with $^{14}C$-phenyl labeled leptophos were conducted with $S_{NAIDM}$ flies treated with 10 µg/g and $R_P$ flies treated with 500 µg/g. The total amount of each metabolite recovered with this label also are presented in Table 12. On the whole, the results are quite similar to those obtained with the $^{14}C$-phenoxy label. With the label in the phenyl ring the various expected derivatives of phenylphosphonic acid were detected and the total percentage of these metabolites are reasonably consistent with the percentage of phenol recovered when the phenoxy label was used.

Conclusion - Overall, the results reveal that the pathways for the metabolism of leptophos are similar in the white mouse, cotton plant, and house flies. The various metabolic products which were obtained are indicated in the scheme below. The pathways are similar to those proposed for other related organophosphorus esters such as bromophos[5] and ronnel[6].

The formation of O-methyl phenylphosphonothioic acid (VI) probably occurs by oxidative dearylation or by esterase catalyzed hydrolysis of leptophos, or a combination similar to that demonstrated with other phosphorothionate insecticides[7,8,9]. The desulfurated acid VII may be formed by several possible mechanisms, (1) by direct oxidative hydrolysis of (IV), (2) oxidative desulfuration of IV to the oxon V followed by hydrolysis, or (3) by desulfuration of the thionic acid VI. The oxidative hydrolysis of phosphorothionate esters

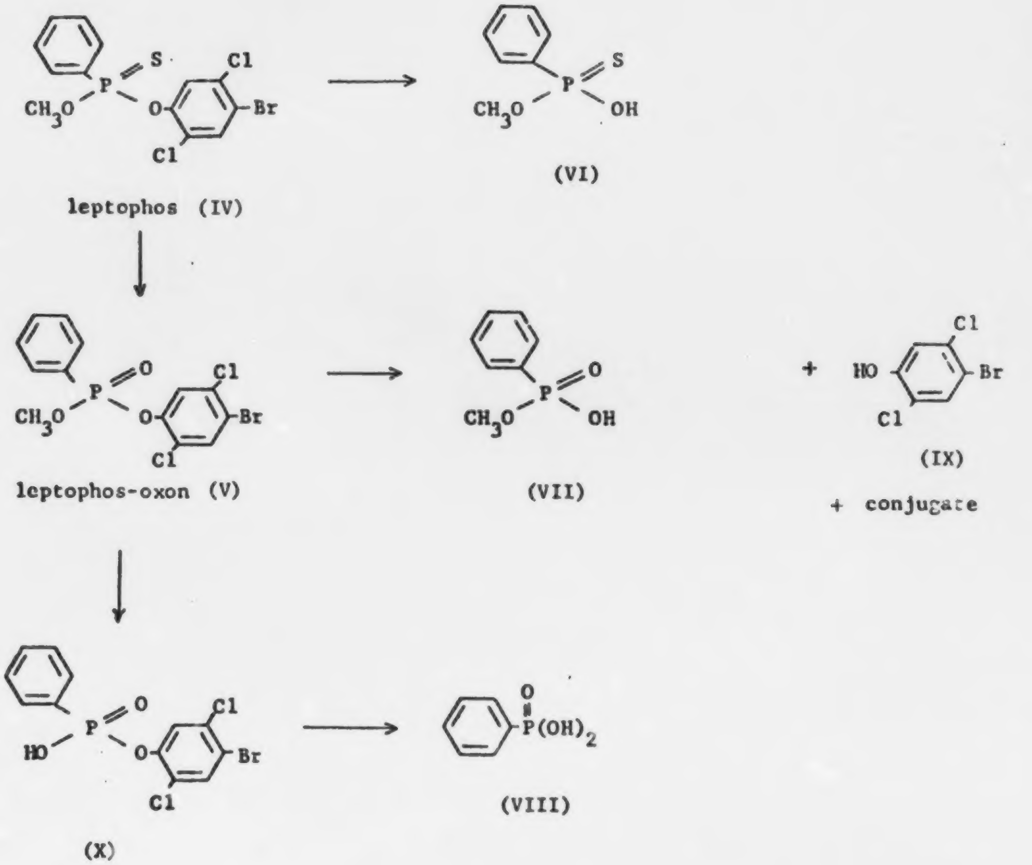

leptophos (IV)
(VI)
leptophos-oxon (V)
(VII)
+
(IX)
+ conjugate
(X)
(VIII)

Table 12. SUMMATION OF METABOLIC PRODUCTS RECOVERED FROM SUSCEPTIBLE AND RESISTANT HOUSEFLIES AFTER TOPICAL APPLICATION WITH RADIOLABELED LEPTOPHOS

| $^{14}$C-Phenoxy leptophos | $R_f$ [b] | Per cent total applied radioactivity[a] | | |
|---|---|---|---|---|
| | | $^S$NAIDM (10 µg/g) | $^R$parathion (10 µg/g) | $^R$parathion (500 µg/g) |
| IV | 0.65 | 81.05<br>(8.11) | 74.24<br>(7.42) | 88.53<br>(442.65) |
| V | 0.25 | 5.15<br>(0.52 | 3.08<br>(0.31) | 3.12<br>(15.60) |
| IX | 0.39 | 4.84<br>(0.48) | 14.50<br>(1.45) | 3.75<br>(18.75) |
| X | 0.13 | 3.48<br>(0.35) | 2.80<br>(0.28) | 3.68<br>(18.40) |
| | Total | 94.52 | 94.62 | 99.08 |
| $^{14}$C-Phenyl leptophos | $R_f$ [c] | | | |
| IV | 0.89 | 91.63<br>(9.16) | - | 84.24<br>(421.20) |
| V | 0.78 | 5.62<br>(0.56) | - | 4.13<br>(20.65) |
| VI | 0.44 | 2.49<br>(0.25) | - | 0.81<br>(4.05) |
| VII | 0.27 | 2.34<br>(0.23) | - | 7.06<br>(35.30) |
| VIII | 0.10 | 2.32<br>(0.23) | - | 0.48<br>(2.40) |
| X | 0.62 | 1.08<br>(0.11 | | 0.36<br>(1.80) |
| | Total | 103.51 | | 97.08 |

---

[a] Parenthetical values represent amounts in µg/g.

[b] Benzene-chloroform (1:1) solvent. [c] Acetonitrile-water-ammonium hydroxide (8:1.8:0.2) solvent.

by microsomal oxidase enzymes to give directly the dialkyl phosphoric acid has been demonstrated by $^{18}O$ studies with parathion[10,11].

Results with the phenoxy-labeled leptophos showed that 4-bromo-2,5-dichlorophenol (or its conjugated form) is the sole degradation product of this part of the leptophos molecule. No evidence was obtained for any hydroxylated or dehalogenated products. Thus, the overall metabolism of leptophos in mice is similar to that of parathion and related insecticides, and no unexpected products were observed. In general, leptophos is metabolized rapidly in the mouse, yielding relatively nontoxic excretion products.

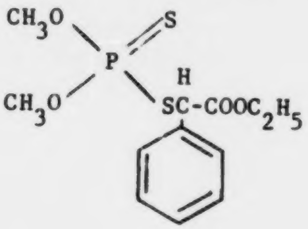
Leptophos evidently penetrates very slowly into the cotton leaf and the term "alteration" is perhaps a better expression than "metabolism" for its behavior on cotton plants. Separate experiments in this laboratory have demonstrated that leptophos virtually has no systemic movement when applied to the stem of cotton leaves, a finding which is consistent with its poor penetration properties. It is difficult to ascertain whether the alteration of leptophos occurs primarily on the surface of the leaf by photochemical processes or within the leaf by plant processes. The evidence indicates that both occur. Nevertheless, the principal component recoverable at all time intervals was leptophos itself, the majority of which was present on the leaf surface.

Penetration of leptophos into resistant house flies is markedly slower than its penetration into susceptible flies. While the difference can account for the tolerance of the resistant strain to low dosages of leptophos, it cannot explain the ineffectiveness of leptophos at high dosages, i.e., as the dosage approaches the $LD_{50}$ level, since substantially larger amounts of leptophos-oxon were recovered from resistant flies treated at 500 μg/g. However, situations similar to this have been observed before with other phosphorothionate insecticides[12,13]. For example, 10-fold more P(O) Phoxim was recovered from resistant flies treated with 150 μg/g Phoxim than from susceptible flies treated with 1.75 μg/g.

The question remains, by what mechanism can resistant house flies tolerate such large amounts of leptophos-oxon? There are several possible explanations. The desulfuration of leptophos to the oxon may occur at a site which is quite remote from the critical lesion and, therefore, the total amount of oxon found in the fly extract does not represent the actual amount of oxon at the target site. It is possible that leptophos or the oxon is partitioned into the fat body of the fly which prevents it from being transported to the target site. Support for this possibility is found in the apparent difficulty encountered in the extraction by organic solvent of leptophos and leptophos-oxon from the internal parts of the flies. In this regard, more recent observations using neurophysiological techniques have revealed that the time required to produce convulsions was slightly less in the susceptible than in the resistant house fly topically treated with leptophos and leptophos-oxon.

### 3. Alteration of O,O-Dimethyl S-[α-(Carboethoxy)benzyl] Phosphorodithioate (Phenthoate) in Citrus, Water, and Upon Exposure to Air and Sunlight

Introduction - Phenthoate (XI) or O,O-dimethyl S-[α-(carboethoxy) benzyl] phosphorodithioate (Cidial®) is an effective broad spectrum organophosphorus insecticide of low mammalian toxicity. Its rat oral LD$_{50}$ is reported to be 4728 mg/kg[14], a value which places it approximately 950-fold less toxic to the rat than to the common house fly, *Musca domestica* L., (LD$_{50}$ 5 mg/kg). Owing to the proven usefulness of phenthoate in controlling citrus scale insects, a study was undertaken to determine the fate and environmental alteration of this material in and on citrus trees. Also examined were the hydrolytic breakdown of phenthoate in an aqueous environment and its photochemical alteration upon exposure to air and sunlight. This section summarizes the results obtained from this study.



phenthoate

Synthesis of Radiolabels and Model Metabolites - Two different radiolabels were used in this study, $^{32}$P and ring-labeled $^{14}$C phenthoate. $^{32}$P phenthoate was prepared by reacting $^{32}$P O,O-dimethyl phosphorodithioic acid (Amersham-Searle) with an equivalent amount of ethyl α-bromophenylacetate. Phenyl ring-labeled $^{14}$C phenthoate was obtained from New England Nuclear Corp. (Boston). Each label was >99% purity as estimated by tlc.

The following compounds were synthesized by unequivocal methods for use as model compounds in the identification of the various metabolites by cochromatography. The structure of all compounds was verified by proton magnetic resonance spectroscopy (pmr), elemental analysis, and in special cases by mass spectroscopy.

XII Phenthoate oxon, O,O-dimethyl S-[α-(carboethoxy)benzyl] phosphorothioate.

XIII Demethyl phenthoate, potassium O-methyl S-[α-(carboethoxy) benzyl phosphorodithioate.

XIV Demethyl phenthoate oxon, sodium *O*-methyl S-[$\alpha$-(carboethoxy)benzyl] phosphorothioate.

XV Phenthoate acid, *O*,*O*-dimethyl *S*-[$\alpha$-(carboxy)benzyl] phosphorodithioate.

XVI Demethyl phenthoate acid, potassium *O*-methyl *S*-[$\alpha$-(carboxy)benzyl phosphorodithioate.

XVII Demethyl phenthoate oxon acid, sodium *O*-methyl *S*-[$\alpha$-(carboxy)benzyl] phosphorothioate.

XVIII *O*,*O*-Dimethyl phosphorodithioic acid.

XIX *O*,*O*-Dimethyl phosphorothioic acid (sodium salt).

XX Ethyl mandelate.

XXII *Bis*-[$\alpha$-(carboxy)benzyl] disulfide.

XXIII *Bis*-[$\alpha$-(carboethoxy)benzyl] disulfide.

XXIV $\alpha$-(Methylthio)phenylacetic acid.

Mandelic acid (XXI) was purchased from Aldrich Chemical Co.

*Metabolism in Citrus* - Selected leaves and fruit of a Valencia orange tree growing in an experimental field plot on this campus were treated by brush application with a solution of 30 mg phenthoate ($^{32}P$ or $^{14}C$) in 50 ml water containing 0.06% emulsifier. This concentration approximated a field application of ½ lb active ingredient per 100 gals of spray solution. Samples were taken at 0, 3, 7, 10, and 14 days after treatment and analyzed.

From the data for total recovery of radioactivity presented in Table 13, it appears that the major portion of phenthoate applied to orange tree leaves is lost by volatilization. The data show a gradual decline in total radioactivity for both labels and after 14 days a reduction of 73% of applied $^{14}C$ phenthoate and approximately 50% of applied $^{32}P$ phenthoate was observed. The rate of decline of total radioactivity recovered from the leaves was greater for the $^{14}C$ label than for the $^{32}P$ label and the source for this difference is attributable to greater loss of radioactivity from the surface of leaves treated with the $^{14}C$ label. It should be noted that the two labels were applied to the orange trees at different times of the year, treatment of $^{14}C$ phenthoate was in March and $^{32}P$ phenthoate was in September, and weather conditions were quite different during these periods.

The amounts of radioactivity detected in the internal fractions, i.e., in the acetone extract and solid residue, were in closer agreement

### Table 13. THE PENETRATION OF $^{14}C$ and $^{32}P$ PHENTHOATE INTO ORANGE LEAVES AND FRUIT, AND THE DISTRIBUTION OF RADIOACTIVITY IN THE SURFACE WASH, ACETONE EXTRACT AND SOLID RESIDUE

| | Percentage of the applied radioactivity recovered at indicated time | | | | | |
|---|---|---|---|---|---|---|
| | 0 | | 3 dy | 7 dy | 10 dy | 14 dy |
| Orange Leaves | | | | | | |
| $^{14}C$ labeled phenthoate | | | | | | |
| External surface wash | 96.7 | (63.3)[a] | 41.8 | 5.0 | 2.1 | 3.0 |
| Internal acetone extract | - | - | 2.4 | 5.5 | 2.6 | 1.8 |
| Solid residue | 3.3 | (2.2) | 16.9 | 20.8 | 15.5 | 22.2 |
| Total recovered | 100.0 | (65.5) | 61.1 | 31.3 | 20.2 | 27.0 |
| $^{32}P$ labeled phenthoate | | | | | | |
| External surface wash | 94.4 | (42.80) | 79.5 | 69.6 | 49.0 | 29.0 |
| Internal acetone extract | - | - | 9.5 | 9.6 | 6.3 | 8.8 |
| Solid residue | 5.6 | (2.54) | 4.5 | 8.0 | 2.5 | 13.0 |
| Total recovered | 100.0 | (45.34) | 93.5 | 87.2 | 57.8 | 50.8 |
| Orange Fruit | | | | | | |
| $^{14}C$ labeled phenthoate | | | | | | |
| External surface wash | 72.5 | (2.14) | 66.1 | 9.8 | 8.1 | 4.6 |
| Internal acetone extract[b] | - | - | 5.4 | 15.6 | 12.8 | 7.8 |
| Solid residue[b] | 27.5 | (0.81) | 21.7 | 21.6 | 34.7 | 28.3 |
| Total recovered | 100.0 | (2.95) | 93.2 | 47.0 | 55.6 | 40.7 |
| $^{32}P$ labeled phenthoate | | | | | | |
| External surface wash | 96.4 | (1.62) | 63.4 | 17.4 | 13.6 | 11.6 |
| Internal acetone extract[b] | - | - | 5.9 | 22.0 | 24.2 | 18.2 |
| Solid residue[b] | 3.6 | (0.06) | 19.4 | 13.9 | 16.3 | 13.2 |
| Total recovered | 100.0 | (1.68) | 88.7 | 53.3 | 54.1 | 43.0 |

[a] ( ) is the amount of phenthoate in ppm wet weight of leaves or fruit.

[b] Includes peel only. Radioactivity in pulp was less than 1% of the applied.

although amounts found in the acetone extracts were consistently larger for the $^{32}$P label while lesser amounts of this label were found in the solid residue.

Analogous data for the recovery of phenthoate radioactivity from orange fruit are presented in Table 14. Significant loss in total recovery also was evident with the fruit but in this case the rates of decline of radioactivity for the two labels were in reasonably close agreement. Noteworthy is the observation that less than 1% of the applied radioactivity was detected in the orange pulp for both $^{14}$C and $^{32}$P labels. Control experiments were conducted to show that very little radioactivity was lost during the work-up.

The various phenthoate metabolites observed in the surface wash and acetone extracts of orange leaves are listed in Tables 15 ($^{14}$C label) and 16 ($^{32}$P label). Qualitatively, the $^{14}$C metabolites isolated from these two fractions are the same. The major compound isolated was unchanged phenthoate (XI). Metabolites present in varying quantities were phenthoate-oxon (XII), demethyl phenthoate (XIII), mandelic acid (XXI), and *bis*-[α-(carboethoxy)benzyl] disulfide (XXIII). Recovered from the $^{32}$P label were XI, XII, *O*,*O*-dimethyl phosphorodithioic adid (XVIII) and *O*,*O*-dimethyl phosphorothioic acid (XIX). The detection of XVIII and XIX from the $^{32}$P label and XXI and XXIII from the $^{14}$C label is consistent with the products expected from the hydrolytic degradation of phenthoate. One unknown of very minor significance was isolated from the $^{14}$C label and two unknowns from the $^{32}$ label, both of which were present in the surface wash.

Tables 16 and 17 provide similar data for the metabolism of $^{14}$C and $^{32}$P phenthoate in orange fruit. Again, as in the case of leaves, unchanged XI was the major product recovered, followed by XII in order of importance. The various other metabolites which were present in orange leaves also were found in fruit.

Alteration in Water - The behavior of phenthoate in water was quite complex and the identity and amounts of the various products isolated after allowing $^{14}$C phenthoate to stand in 0.01 *M* phosphate buffer at pH 6, 7, and 8 for intervals of up to 28 days are presented in Table 18. Evidently, phenthoate is fairly stable in water at the indicated pH values. Decomposition appeared to be fastest at pH 8 and the rate of decomposition was first-order with respect to phenthoate. At this pH, the calculated half-life of phenthoate was approximately 12 days.

Of the various hydrolytic products, phenthoate acid (XV) was observed in greatest abundance, irrespective of pH, indicating that the carboethoxy moiety was the most vulnerable part of the molecule to hydrolytic decomposition. The rate of formation of XV was significantly slower at pH 6 and, in spite of the obvious experimental variation in

Table 14. AMOUNT OF $^{14}C$ METABOLITES FOUND IN THE SURFACE WASH AND ACETONE EXTRACT OF ORANGE LEAVES AFTER INDICATED TIME INTERVALS

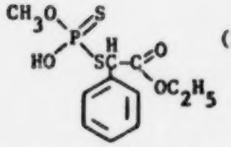
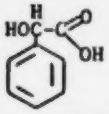
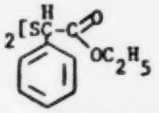
| Compound found in indicated material | Percent of recovered radioactivity after indicated time | | | |
|---|---|---|---|---|
| | 3 dy | 7 dy | 10 dy | 14 dy |
| Phenthoate (XI) | | | | |
| Surface wash | 84.4 | 45.5 | 35.4 | 41.6 |
| Acetone extract | 4.6 | 46.1 | 37.7 | 17.0 |
| Phenthoate oxon (XII) | | | | |
| Surface wash | 0 | 0 | 2.8 | 5.1 |
| Acetone extract | 0.2 | 0 | 7.2 | 12.4 |
|  (XIII) | | | | |
| Surface wash | 0 | 0.2 | 0.9 | 2.0 |
| Acetone extract | 0 | 0 | 4.0 | 2.5 |
|  (XXI) | | | | |
| Surface wash | 0 | 0.1 | 1.3 | 3.6 |
| Acetone extract | 0.6 | 6.2 | 6.4 | 5.7 |
|  (XXIII) | | | | |
| Surface wash | 10.2 | 1.5 | 4.3 | 9.7 |
| Acetone extract | 0 | 0 | 0 | 0 |
| Unknown I | | | | |
| Surface wash | 0 | 0.4 | 0 | 0.4 |
| Acetone extract | 0 | 0 | 0 | 0 |
| Total | 100 | 100 | 100 | 100 |

Table 15. AMOUNT OF $^{32}P$ METABOLITES FOUND IN THE SURFACE WASH AND ACETONE EXTRACT OF ORANGE LEAVES AT THE INDICATED TIME

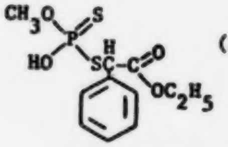
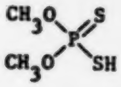
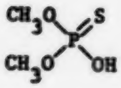
| Compound found in indicated material | Percent of recovered material at the indicated time | | | |
|---|---|---|---|---|
| | 3 dy | 7 dy | 10 dy | 14 dy |
| Phenthoate (XI) | | | | |
| Surface wash | 63.4 | 39.1 | 52.1 | 43.5 |
| Acetone extract | 9.4 | 10.3 | 7.2 | 14.9 |
| Phenthoate oxon (XII) | | | | |
| Surface wash | 12.8 | 34.4 | 22.8 | 17.6 |
| Acetone extract | 0.9 | 1.0 | 2.6 | 4.5 |
|  (XIII) | | | | |
| Surface wash | 2.4 | 4.1 | 4.6 | 4.2 |
| Acetone extract | 0 | 0 | 0 | 0 |
|  (XVIII) | | | | |
| Surface wash | 7.2 | 4.1 | 0 | 4.5 |
| Acetone extract | 0.4 | 0.8 | 0 | 3.4 |
|  (XIX) | | | | |
| Surface wash | 0 | 0 | 4.2 | 3.4 |
| Acetone extract | 0 | 0 | 1.6 | 0.5 |
| Unknown I | | | | |
| Surface wash | 3.5 | 1.1 | 4.9 | 3.5 |
| Acetone extract | 0 | 0 | 0 | 0 |
| Unknown II | | | | |
| Surface wash | 0 | 5.1 | 0 | 0 |
| Acetone extract | 0 | 0 | 0 | 0 |
| Total | 100 | 100 | 100 | 100 |

Table 16. AMOUNT OF $^{14}C$ METABOLITES FOUND IN THE SURFACE WASH AND ACETONE EXTRACT OF ORANGE PEELS AT THE INDICATED TIME

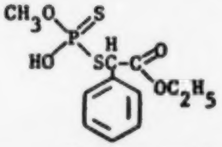
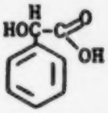
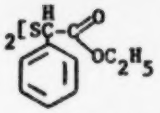
| Compound found in indicated material | Percent of recovered radioactivity at indicated time | | | |
|---|---|---|---|---|
| | 3 dy | 7 dy | 10 dy | 14 dy |
| Phenthoate (XI) | | | | |
| Surface wash | 90.3 | 31.3 | 37.9 | 35.6 |
| Acetone extract | 7.4 | 52.5 | 54.7 | 58.2 |
| Phenthoate oxon (XII) | | | | |
| Surface wash | 0 | 0 | 0 | 0.5 |
| Acetone extract | 0.2 | 2.0 | 2.2 | 2.2 |
|  (XIII) | | | | |
| Surface wash | 0 | 0.7 | 0.3 | 0.2 |
| Acetone extract | 0 | 5.8 | 2.3 | 0 |
|  (XXI) | | | | |
| Surface wash | 0 | 0.8 | 0 | 0.2 |
| Acetone extract | 0 | 1.5 | 2.1 | 2.3 |
|  (XXIII) | | | | |
| Surface wash | 2.1 | 5.2 | 0.5 | 0.7 |
| Acetone extract | 0 | 0 | 0 | 0 |
| Unknown I | | | | |
| Surface wash | 0 | 0.2 | 0 | 0.1 |
| Acetone extract | 0 | 0 | 0 | 0 |
| Total | 100 | 100 | 100 | 100 |

Table 17. AMOUNT OF $^{32}P$ METABOLITES FOUND IN THE SURFACE WASH AND ACETONE EXTRACT OF ORANGE PEELS AT THE INDICATED TIME.

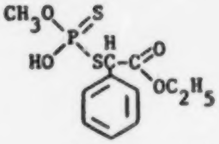
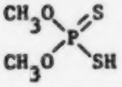
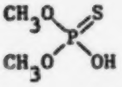
| Compound found in indicated material | Percent of recovered material at the indicated time | | | |
|---|---|---|---|---|
| | 3 dy | 7 dy | 10 dy | 14 dy |
| Phenthoate (XI) | | | | |
| Surface wash | 74.0 | 33.3 | 24.6 | 23.7 |
| Acetone extract | 8.5 | 39.0 | 42.9 | 22.1 |
| Phenthoate oxon (XII) | | | | |
| Surface wash | 12.7 | 6.1 | 6.3 | 7.8 |
| Acetone extract | 0 | 9.5 | 5.3 | 4.0 |
|  (XIII) | | | | |
| Surface wash | 0 | 0 | 0 | 0 |
| Acetone extract | 0 | 7.4 | 0 | 0 |
|  (XVIII) | | | | |
| Surface wash | 0 | 1.7 | 1.5 | 2.9 |
| Acetone extract | 0 | 0 | 12.2 | 13.2 |
|  (XIX) | | | | |
| Surface wash | 0 | 2.3 | 0 | 3.0 |
| Acetone extract | 0 | 0 | 3.6 | 6.5 |
| Unknown I | | | | |
| Surface wash | 4.8 | 0.7 | 2.2 | 1.6 |
| Acetone extract | 0 | 0 | 0 | 15.2 |
| Unknown II | | | | |
| Surface wash | 0 | 0 | 1.4 | 0 |
| Acetone extract | 0 | 0 | 0 | 0 |
| Total | 100 | 100 | 100 | 100 |

Table 18. THE BREAKDOWN PRODUCTS OF $^{14}$C LABELED PHENTHOATE IN 0.01 M SODIUM PHOSPHATE BUFFER AT pH 6, 7, and 8 AFTER THE INDICATED TIME. (TEMPERATURE 24.5°C)

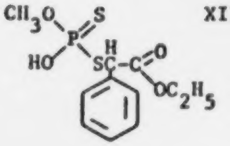
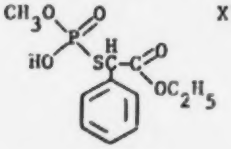
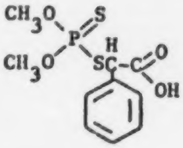
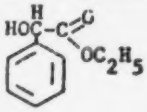
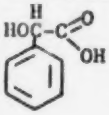
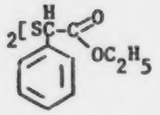
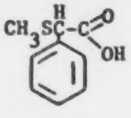
| Compound | pH | Percent of recovered radioactivity after indicated time | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.25 dy | 0.5 dy | 1 dy | 2 dy | 3 dy | 7 dy | 14 dy | 21 dy | 28 dy |
| Phenthoate XI | 6 | 98.9 | 96.9 | 96.6 | 95.9 | 98.7 | 95.7 | 55.6 | 57.0 | 45.1 |
| | 7 | 97.2 | 95.1 | 97.7 | 91.6 | 83.6 | 75.6 | 26.7 | 44.3 | 21.3 |
| | 8 | - | 92.0 | 89.2 | 84.0 | 81.6 | 39.5 | 54.5 | 31.6 | 22.2 |
|  XIII | 6 | 0 | 0 | 0 | 0.7 | 0.3 | 0.6 | 10.6 | 15.7 | 18.6 |
| | 7 | 0 | 0.3 | 0.2 | 0.8 | 4.7 | 2.8 | 7.1 | 7.3 | 4.1 |
| | 8 | - | 0.6 | 0.4 | 0.3 | 0 | 0 | 2.0 | 3.2 | 7.4 |
|  XIV | 6 | 0 | 0 | 0 | 1.9 | 0.5 | 1.8 | 4.9 | 0.9 | 1.0 |
| | 7 | 0 | 0.6 | 0 | 0 | 4.9 | 3.6 | 13.1 | 9.0 | 7.3 |
| | 8 | - | 2.2 | 1.0 | 1.1 | 5.5 | 16.1 | 7.7 | 9.5 | 11.2 |
|  XV | 6 | 0 | 0.8 | 1.7 | 0.7 | 0.5 | 1.2 | 28.9 | 22.7 | 30.4 |
| | 7 | 0.5 | 0.5 | 0.5 | 2.1 | 6.8 | 16.7 | 46.5 | 35.7 | 55.8 |
| | 8 | - | 3.5 | 5.1 | 9.8 | 12.9 | 31.9 | 32.6 | 46.5 | 50.3 |
| Combined compounds XVI and XVII | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.3 | 3.3 |
| | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 2.7 | 1.3 | 4.2 |
| | 8 | - | 0 | 0 | 0 | 0 | 0 | 0 | 1.6 | 1.6 |
|  XX | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 7 | 0 | 0.3 | 0.2 | 0 | 0 | 0 | 0 | 0.7 | 0 |
| | 8 | - | 0 | 0 | 0 | 0 | 0 | 0 | 2.3 | 0.7 |
|  XXI | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 7 | 0 | 0 | 0.3 | 1.7 | 0 | 0 | 0 | 0 | 0 |
| | 8 | - | 0 | 0 | 0 | 0 | 0 | 1.4 | 2.1 | 1.3 |

Table 18. continued.

| Compound | pH | Percent of recovered radioactivity after indicated time | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 0.25 dy | 0.5 dy | 1 dy | 2 dy | 3 dy | 7 dy | 14 dy | 21 dy | 28 dy |
|  XXIII | 6 | 1.1 | 2.3 | 1.7 | 0.8 | 0 | 0 | 0 | 0 | 0 |
| | 7 | 2.3 | 3.3 | 1.1 | 3.8 | 0 | 0 | 0 | 0 | 0 |
| | 8 | - | 1.6 | 4.2 | 4.2 | 0 | 0 | 0 | 0 | 0 |
|  XXIV | 6 | 0 | 0 | 0 | 0 | 0 | 0.7 | 0 | 1.4 | 1.6 |
| | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 1.9 | 0.5 | 0 |
| | 8 | - | 0 | 0 | 0 | 0 | 12.6 | 1.8 | 3.0 | 1.1 |
| | | | | | | | | | | |
| Unknown I | | 0 | 0 | 0 | 0 | 0 | 1.4[a] | 0.7[a] | 1.3[a] | 2.1[a] |
| Unknown II | | 0 | 0 | 0 | 0 | 0 | 0 | 1.2[a] | 0 | 0 |
| Unknown III | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2.7[b] |
| Unknown IV | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5.2[a]<br>1.4[b] |

[a] Unknown found at pH 7.0.

[b] Unknown found at pH 8.0.

the data, hydrolysis of the carboethoxy moiety appeared to be fastest at pH 8.0. On the other hand, the rate of formation of demethyl phenthoate (XIII), the second most prominent hydrolysis product, was fastest at pH 6.

Significant amounts of demethyl phenthoate oxon (XIV) were detected, particularly at pH 7 and 8. Since at no time was any phenthoate oxon (XII) noted, it is most likely that XIV was formed from XIII, possibly by hydrolytic desulfuration. Other products observed in small amounts were mandelic acid (XXI), bis-[$\alpha$-(carboethoxy)benzyl] disulfide (XXIII), $\alpha$-(methylthio)phenylacetic acid (XXIV), demethyl phenthoate acid (XVI) and demethyl phenthoate oxon acid (XVII). Several unknowns of minor significance also were detected, particularly after standing for long time intervals.

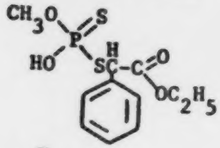
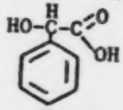
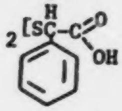
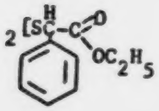
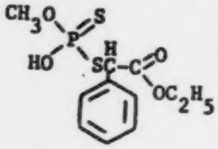
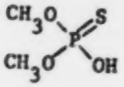
A single study of the hydrolysis of $^{32}$P phenthoate was conducted at pH 8. The products from the $^{32}$P label largely supported results from the C$^{14}$ label. XV was confirmed as the principal hydrolytic product, followed by XIII and XIV. Other products included XVI, XVII, O,O-dimethyl phosphorodithioic acid (XVIII), and O,O-dimethyl phosphorothioic acid (XIX).

Photodegradation - The photoalteration of $^{14}$C and $^{32}$P phenthoate in sunlight under atmospheric conditions was examined as thin films on glass plates. Air temperatures during the exposure period ranged from 23.3° to 7.2°C (typical day in March). Under these conditions phenthoate was quite volatile and approximately 90% of the applied material was lost after 40 hours of exposure. The high rate of loss of phenthoate from a glass surface adds support to an earlier conclusion that the principal route for the loss of phenthoate from orange leaves and fruit was through volatilization.

The products recovered from the glass plates for both labels are given in Table 19. The principal constituent present in the residue was unchanged phenthoate which gradually decreased in proportion to the other alteration products during the period of exposure. Phenthoate oxon (XII) also was a significant product and the material increased in amount as time progressed. Thus, after 35-hr exposure to sunlight, 19% and 28% of the residual material consisted of XII derived from $^{14}$C and $^{32}$P phenthoate, respectively. Other products isolated were demethyl phenthoate (XIII) with both labels; mandelic acid (XXI), bis-[$\alpha$-(carboxy)benzyl] disulfide (XXII), and bis-[$\alpha$-(carboethoxy)benzyl] disulfide (XXIII) from the $^{14}$C label; and O,O-dimethyl phosphorothioic acid XIX from the $^{32}$P label.

Conclusion - Based on this study, the proposed pathways for the alteration of phenthoate in orange leaves and fruit, in water, and upon exposure to sunlight and the atmosphere are presented in Figure 3. In gross aspects, the alteration scheme is similar to those reported for phenthoate metabolism in cabbage, apples, and strawberries[15].

Table 19. THE METABOLITES OF $^{14}$C and $^{32}$P PHENTHOATE APPLIED TO THE SURFACE OF GLASS PLATES AND EXPOSED TO SUNLIGHT

| Compound found at indicated time | | Percent of recovered radioactivity after indicated time of exposure | | | | | |
|---|---|---|---|---|---|---|---|
| | | 7 hr | 14 hr | 21 hr | 28 hr | 35 hr | 42 hr |
| $^{14}$C phenthoate | | | | | | | |
| Phenthoate | (XI) | 92.2 | 84.4 | 89.5 | 76.9 | 71.7 | |
| Phenthoate oxon | (XII) | 3.7 | 8.8 | 5.3 | 11.7 | 19.8 | |
|  | (XIII) | 2.1 | 2.7 | 1.5 | 4.3 | 1.9 | |
|  | (XXI) | 0 | 0.6 | 0.7 | 1.0 | 2.2 | |
|  | (XXII) | 0 | 0.8 | 1.5 | 2.0 | 2.5 | |
|  | (XXIII) | 2.0 | 2.7 | 1.5 | 4.1 | 1.9 | |
| Total | | 100 | 100 | 100 | 100 | 100 | |
| $^{32}$P phenthoate | | | | | | | |
| Phenthoate | (XI) | 84.5 | 76.8 | 61.7 | 51.1 | 51.1 | 35.5 |
| Phenthoate oxon | (XII) | 13.4 | 15.2 | 30.0 | 29.2 | 28.0 | 35.4 |
|  | (XIII) | 0 | 3.0 | 3.1 | 5.2 | 10.6 | 6.3 |
|  | (XIX) | 2.1 | 5.0 | 5.2 | 14.5 | 10.3 | 6.1 |
| Unknown | | 0 | 0 | 0 | 0 | 0 | 16.7 |
| Total | | 100 | 100 | 100 | 100 | 100 | 100 |

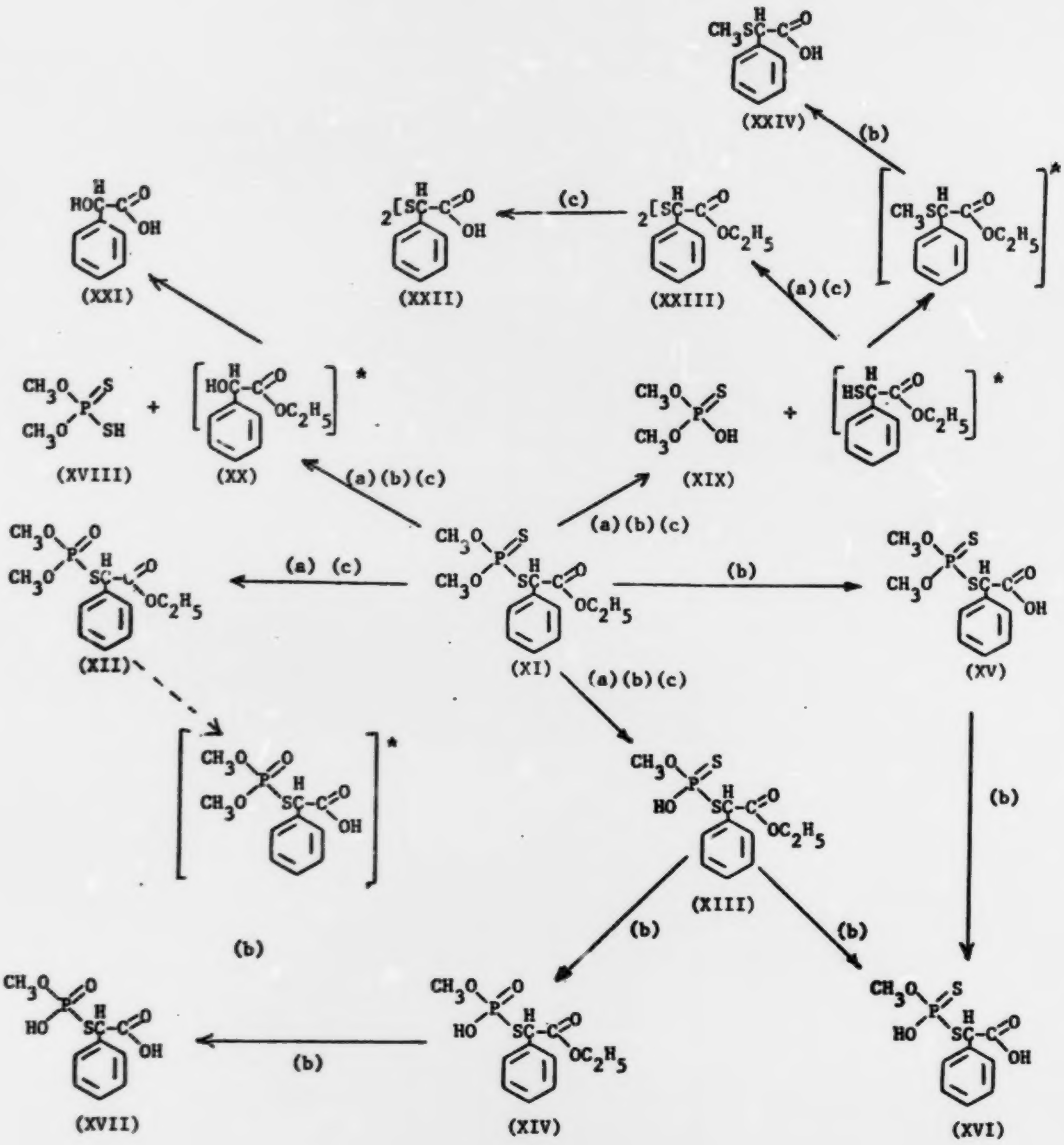
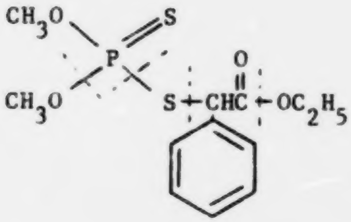

(XXIV)
(b)
(XXI)
(XXII)
(c)
(XXIII)
(a)(c)
(XVIII)
(XX)
(a)(b)(c)
(XIX)
(a)(b)(c)
(XII)
(a) (c)
(XI)
(b)
(XV)
(a)(b)(c)
(XIII)
(b)
(b)
(b)
(b)
(XVII)
(b)
(XIV)
(XVI)
*
*
*
*

Fig. 3. Proposed alteration pathways for phenthoate (a) in citrus, (b) in buffered water, and (c) after exposure to sunlight. * denotes hypothetical intermediate that is not isolated.

Also, the proposed scheme is similar in many respects to that reported for the metabolism of dimethoate [O,O-dimethyl S-(N-methylcarbamoylmethyl) phosphorodithioate], a compound of closely related structure, in a variety of plants.[16]

As indicated in the scheme, the initial step in the degradation of phenthoate is hydrolytic in nature. The various places in the molecule which are vulnerable to hydrolytic cleavage are indicated (below) by the dotted lines. Evidently, reactions involving each of these bonds take place, giving rise to the complex array of products.



Oxidative reactions also occurred, as evident by the formation of phenthoate oxon (II) by desulfuration and the coupling reaction leading to the disulfides XII and XIII.

According to the scheme the alteration products of phenthoate in citrus and upon exposure to sunlight and air on glass plates are essentially the same. Thus, it appears that the formation of the various products recovered from the surface of orange leaves and fruit is attributable to environmental effects. Products found internally in citrus are probably the result of both biological and nonbiological transformation since alteration products generated on the surface undoubtedly penetrate into the leaves or the fruit.

It has been speculated that oxidants in air pollution may have a significant effect on the alteration of pesticides in the environment.[17] Therefore, there is a possibility that some of the discrepancies observed in this study, particularly in citrus and on glass plates, may be accountable by effects from photochemical smog. For example, the relative amounts of phenthoate oxon which were recovered from citrus (surface wash) and glass plates were substantially greater for the $^{32}P$ than for the $^{14}C$ label. The studies with the $^{32}P$ label were conducted in August-September, a period when air pollution levels are high, while the $^{14}C$ studies were conducted during months (March-April) which are relatively free of air pollution. While definite conclusions cannot be reached from this study owing to the absence of proper controls, the results cited above suggest that oxidants present in air pollution contribute to the oxidative desulfuration reaction.

Under near neutral pH conditions the most susceptible linkage in the phenthoate molecule to hydrolysis appears to reside in the carboethoxy moiety and not with linkage associated with the phosphorus atom. Although comparable studies under similar conditions have not been conducted with malathion (diethyl mercaptosuccinate S-ester with O,O-dimethyl phosphorodithioate) and other organophosphorus insecticides which contain carbonyl ester moieties, under strongly basic or acid conditions malathion has been reported to hydrolyze to O,O-dimethyl phosphorodithioic acid and diethyl fumarate, and to O,O-dimethyl phosphorothioic acid and diethyl 2-mercaptosuccinate, respectively[18]. Thus, malathion undergoes C-S bond cleavage in base, probably through a β-elimination reaction, and P-S bond cleavage in acid. While direct comparison with phenthoate cannot be made owing to the different conditions employed, the two compounds appear to differ in the relative susceptibility of their respective carboethoxy moieties to hydrolytic attack.

In general, the various alteration products which were isolated and identified in this study represent compounds which would normally be anticipated from the structure of phenthoate. With the exception of phenthoate oxon, all of the alteration products represent materials which may be regarded as detoxication products. Because of its very low acute mammalian toxicity, high susceptibility to environmental degradation, and general effectiveness against a variety of insect pests, phenthoate should prove to be a useful insecticide of minimum threat to public health and the environment.

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## D. BASIC STUDIES ON THE INHIBITION OF THE CHOLINESTERASE ENZYMES

A number of investigations were completed on the basic aspects of the inhibition of cholinesterase enzymes derived from mammalian and insect sources. These studies were aimed at the elucidation of the fundamental mechanisms involved in cholinesterase inhibition and reactivation involving carbamate and organophosphorus esters.

### 1. The Reactivation of Carbamate Inhibited Cholinesterase

Introduction - The inactivation of acetylcholinesterase (AChE) by esters of methyl- or dimethylcarbamic acid has been demonstrated beyond reasonable doubt to be the result of an actual chemical reaction between the enzyme and ester as shown by the equation below.

$$E + IX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EIX \xrightarrow[X]{k_2} EI \xrightarrow{k_3} E + P \qquad (1)$$

IX is the carbamate ester, E is free AChE, X is the leaving group, EIX is the reversible complex, EI is carbamylated enzyme, and P is alkylcarbamic acid.

During the assay of AChE activity *in vitro* the primary form of inhibited enzyme is most probably the carbamylated enzyme which is responsible for the eventual return of enzymatic activity after the effect of the residual carbamate has been minimized or removed by dilution or gel filtration.

Previous studies on the spontaneous reactivation of AChE inhibited by carbamate esters were carried out by means of dilution techniques and in the absence of the substrate acetylcholine (or acetylthiocholine).[1] In the present study, the carbamylated AChE was separated from excess inhibitor by Sephadex gel filtration and decarbamylation of the inhibited enzyme was determined in the presence of the substrate acetylthiocholine. The effect of different conditions of pH, salt concentrations and temperature were determined in an attempt to elucidate the mechanism of the decarbamylation process.

Carbamate Inhibitors - The carbamates used are indicated as follows: 3-isopropylphenyl methylcarbamate (I), 3-isopropyl-4-nitrophenyl dimethylcarbamate (II), ring-labeled tritiated carbofuran (III) and carbonyl-labeled $^{14}C$ Banol (IV) (6-chloro-3,4-xylyl methylcarbamate).

Kinetic Procedure - After the residual carbamate was removed from the AChE by Sephadex gel filtration, an aliquot was added to the reaction cuvette and hydrolysis of the substrate was monitored according to the procedure of Ellman et al.[2] at 412 mμ. The $k_3$ step indicates that decarbamylation will result in a first-order increase in AChE (or a decrease in carbamylated enzyme). Since the amount of AChE present in successive time intervals is linearly related to the change in absorbance at 412 mμ, it follows that as AChE concentration increases substrate hydrolysis should accelerate and the plot of absorbance versus time should have a positive curvature. The plot would have the form of an integrated first-order equation, i.e.,

$$y = At - A/k(1 - e^{-kt}), \qquad (2)$$

where y is absorbance at time t, A is the rate when all the enzyme is in the uninhibited form, and k is equal to the constant of acceleration or the regeneration rate constant. The value of A, the limiting velocity for ATCh hydrolysis, was estimated from the absorbance versus time plot after the curvilinear line became linear. In all experiments the regeneration reaction was followed until this relationship was linear with time, i.e., when no acceleration component in the curve was detectable.

The data also may be fitted to the following multicompartmental mathematical model and this model was employed in this study to obtain the decarbamylation constant ($k_r$), which is identical to k in Eq. (2).

$$\begin{tikzcd} & & (D) \arrow[r, "k_a'"] & (E) \arrow[d, "k_s'"] \\ (A) \arrow[r, "k_r"] & (B) \arrow[r, "k_a"] & (C) \arrow[r, "k_s"] & (F) \end{tikzcd} \qquad (3)$$

In this model, compartment (A) is inhibited enzyme, (B) is free enzyme, (C) and (E) are the coupled substrated reactions, (D) is free enzyme at $t = 0$, and (F) is optical density. The appropriate rate constant for regeneration of free enzyme is $k_r$, while the $k_a$ and $k_a'$ are indicative of the reaction of this substrate (ATCh) and the $k_s$ and $k_s'$ are charcteristic of the reaction of this substrate hydrolysis product and the reagent DTNB. The data fitting to the model was done utilizing the systems analysis and modeling program of Berman and Weiss[3,4]. In this least-squares program, upper and lower limits for $k_r$ were set by employing values selected from previous studies, e.g., Reiner and

Aldridge[1]. The values so chosen were 0.001 min$^{-1}$ and 0.300 min$^{-1}$ for the lower and upper limits, respectively, for both carbamate derivatives of AChE.

Sephadex Gel Filtration Using Radioisotopes - Since this was a study of the regeneration reaction ($k_3$ or $k_r$), it was of fundamental importance to be sure that the carbamylated enzyme was being isolated from noncovalently bound carbamate. The crucial point, therefore, was to determine the proper functioning of the Sephadex column. The first control experiment was to ascertain the volume that would precede the protein fraction when a 1.0-ml sample was applied. The position of the protein was readily determined by the addition of the dye, Dextran blue, which is excluded from all Sephadex gels and, therefore, flows with the protein. The enzymatic activity of this fraction was assayed by withdrawing an aliquot and adding it to a reaction mixture. One-milliliter samples were collected starting at the first appearance of blue color, the color continuing for four successive milliliters of eluant. The majority of the activity occurred in the first few milliliters after appearance of the color. This experiment indicates two important points. First, the passage through the column apparently does not alter the activity of the enzyme and second, the enzymatic activity is associated with the Dextran blue fraction, as was expected.

The enzymatic scheme (Eq. 1) indicates that the leaving group (X) should be lost from the enzyme during the inhibition process. This was checked by using carbonyl-labeled $^{14}C$ Banol (IV) and ring-labeled 4,5,6-$H^3$-carbofuran (III). When Banol was used as an inhibitor, radioactivity was associated with the enzyme fraction; however, when carbofuran was used, no radioactivity appeared in the enzyme fraction. This provided evidence that the carbamylated enzyme was being isolated.

pH-Rate Dependence - The accepted role of carbamates as alternate substrates for AChE predicts that the decarbamylation reaction might have the same pH-rate profile as noninhibited substrate hydrolysis. The substrate here, acetylthiocholine (ATCh), has been shown[5] to have a pH-rate profile which gives a plateau at high pH in contrast to the bell-shape curve found in ACh hydrolysis. The plateau was not observed in this study.

The pH-rate profiles for decarbamylation of a methyl- and dimethylcarbamyl bovine erythrocyte AChE inhibited by 3-isopropylphenyl methylcarbamate (I) and 3-isopropyl-4-nitrophenyl dimethylcarbamate (II) appear in Figs. 1 and 2 respectively. The maximum rate is found between pH 8.6 and 8.9 for both of the figures. The corresponding regeneration of methyl- and dimethylcarbamyl derivatives of fly-head AChE appear in Figs. 3 and 4 respectively. In this case the methylcarbamyl derivative has a maxima between pH 8.0 and 8.5 and the dimethylcarbamyl enzyme at pH 8.3-8.8. It may be noted that the regeneration rate constant for the methylcarbamyl AChE has a more precipitous decline with increasing pH for both the bovine and house fly enzyme than do the dimethylcarbamyl AChE derivatives.

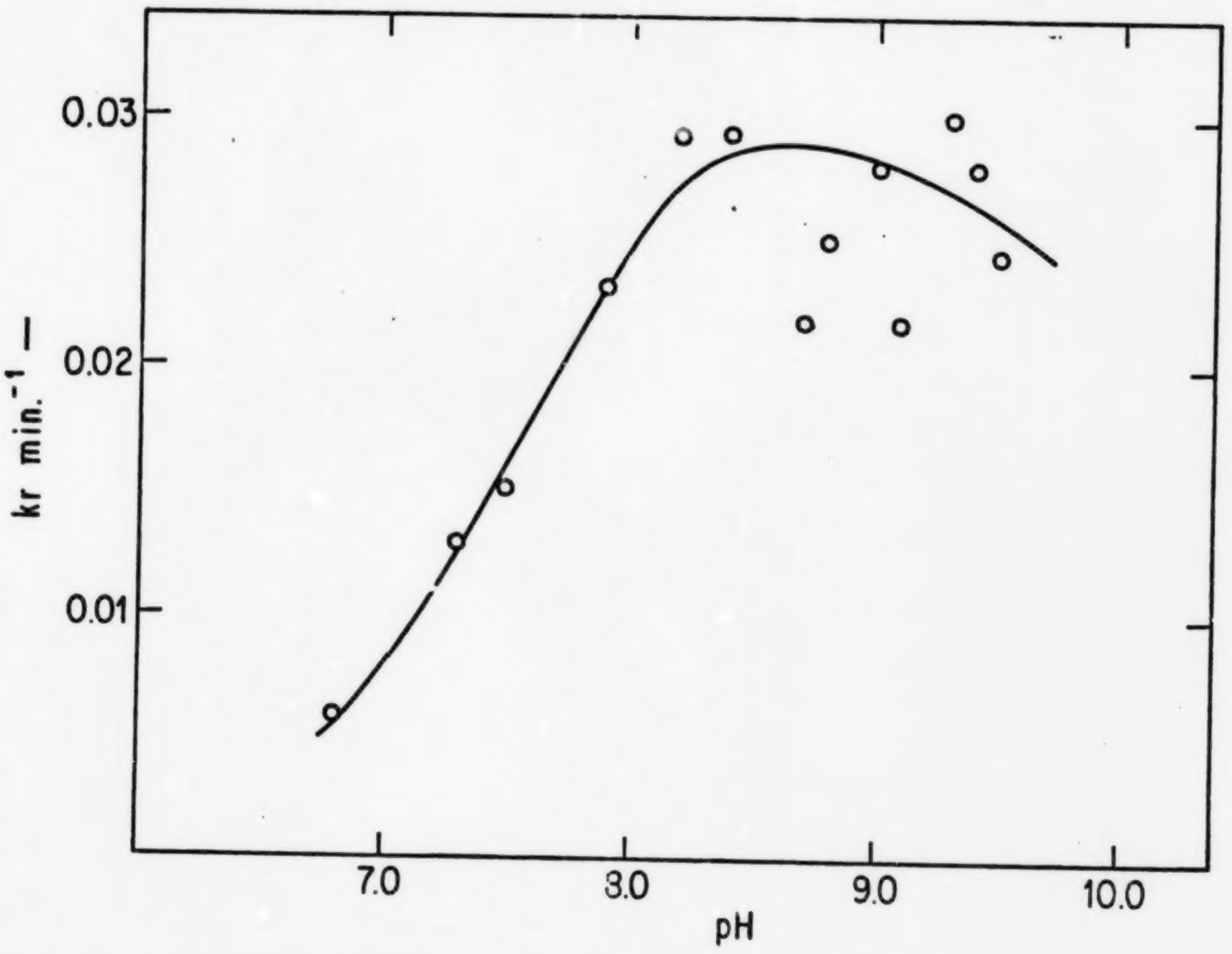


Figure 1. EFFECT OF pH ON THE DECARBAMYLATION RATE CONSTANT OF BOVINE ERYTHROCYTE AChE INHIBITED BY I.

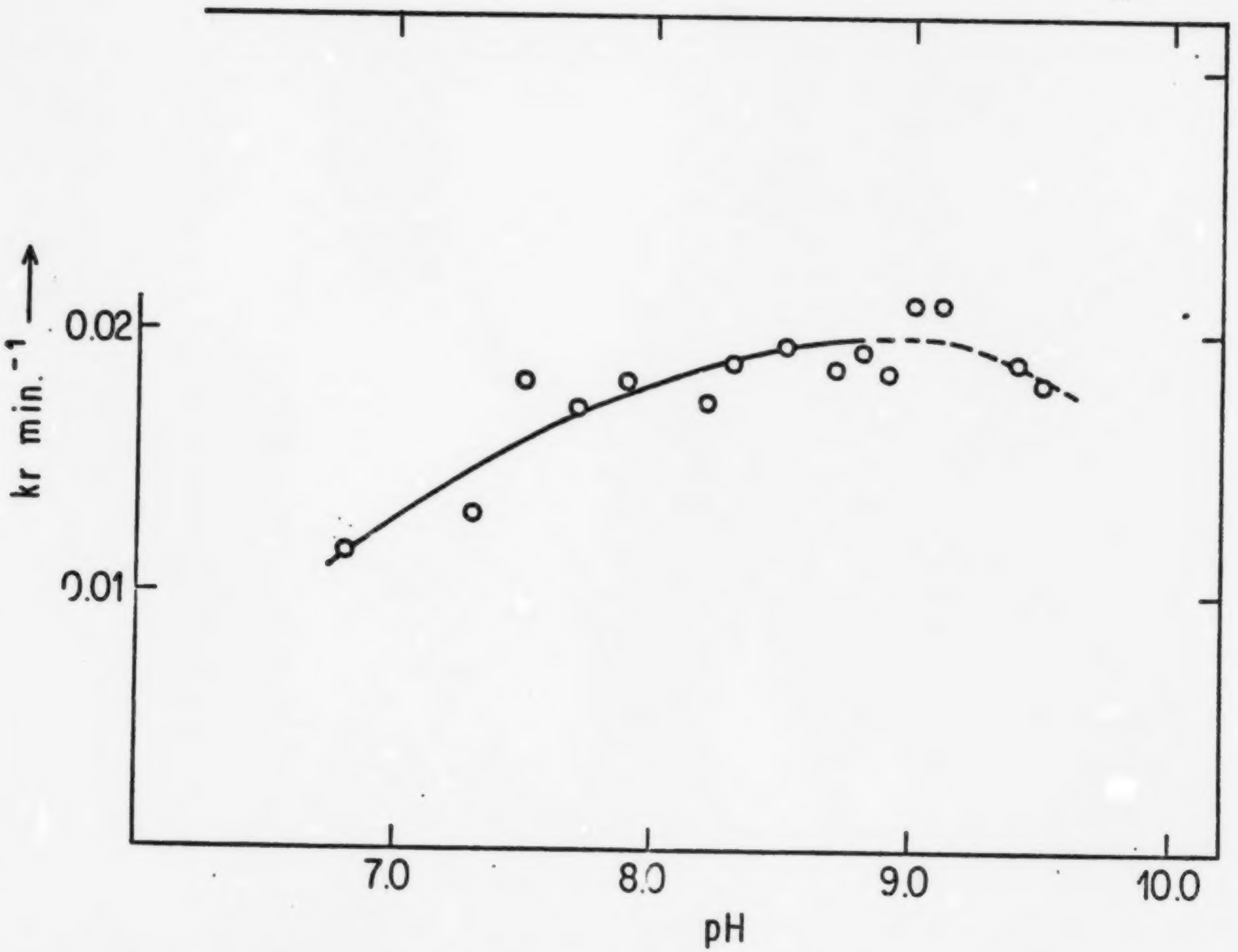


Figure 2. EFFECT OF pH ON THE DECARBAMYLATION RATE CONSTANT OF BOVINE ERYTHROCYTE AChE INHIBITED BY II

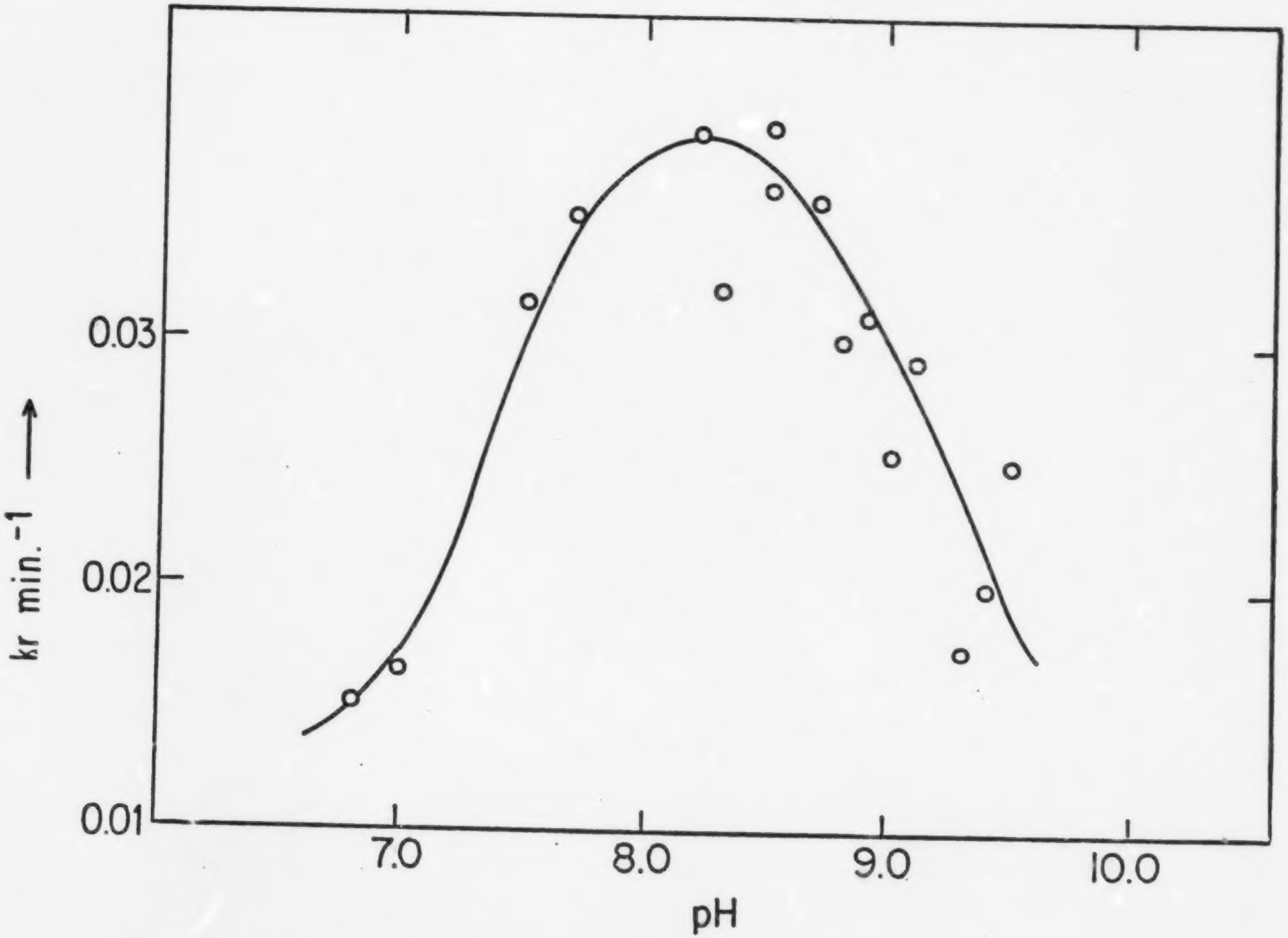


Figure 3. EFFECT OF pH ON THE DECARBAMYLATION RATE CONSTANT FOR HOUSE FLY-HEAD AChE INHIBITED BY I

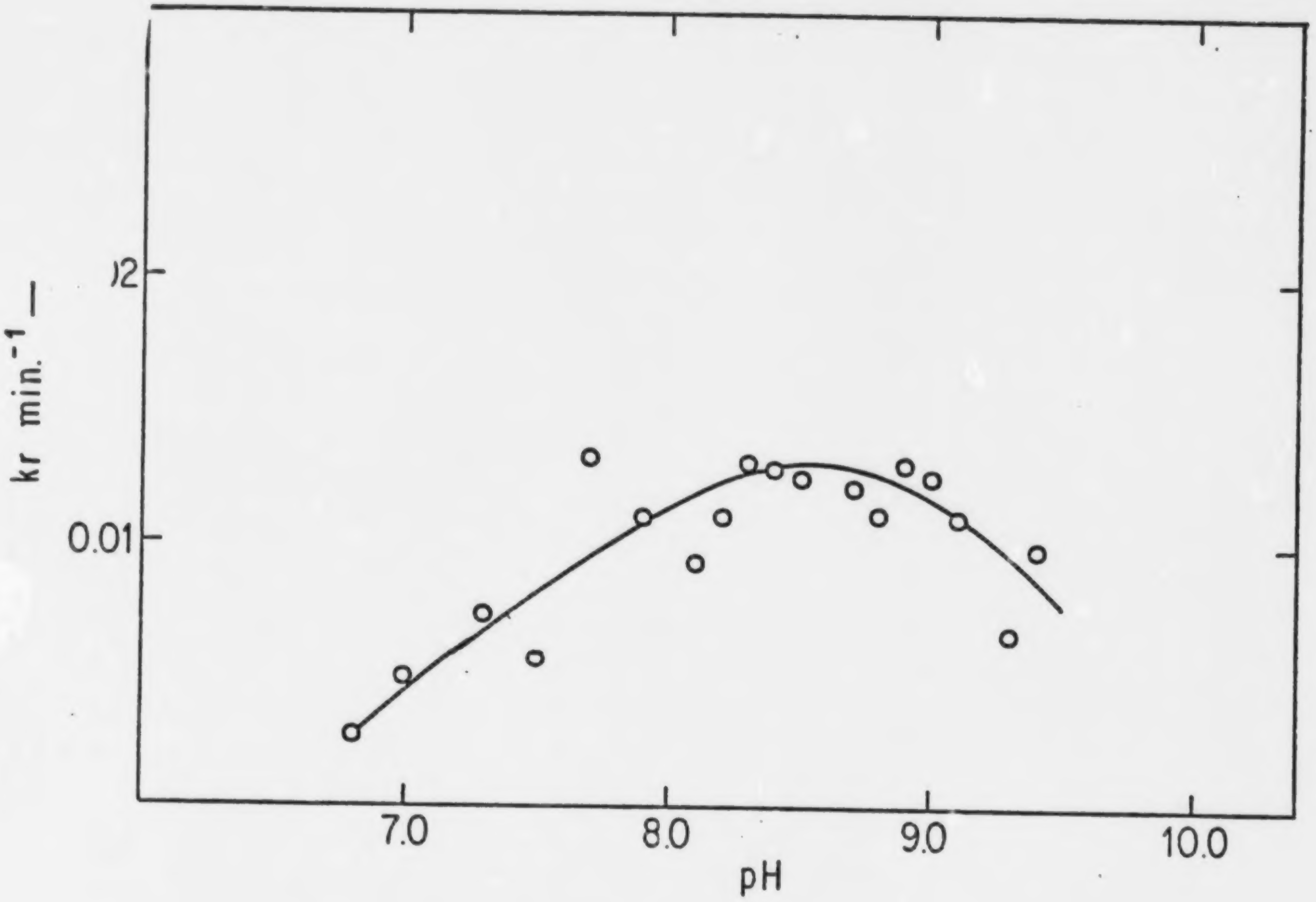


Figure 4. EFFECT OF pH ON THE DECARBAMYLATION RATE CONSTANT FOR HOUSE FLY-HEAD AChE IHIBITED BY II

Activation Parameters - Data showing the effect of temperature on the rate constant for regeneration of house fly and bovine erythrocyte AChE inhibited by 3-isopropylphenyl methylcarbamate (I) and 3-isopropyl-4-nitrophenyl dimethylcarbamate (II) are presented in Table 1. Included in the table are the values for the Arrhenius activation energy ($E_a$) calculated by regression analysis of a plot of log $k_r$ vs 1/T (°K), and values for the entropy of activation ($\Delta S^{\pm}$). Satisfactory Arrhenius plots were obtained with correlation coefficients ranging from 0.94 to 0.99.

Values for the regeneration constants ($k_r$) show that the rate of regeneration of house fly AChE inhibited by I or II is about twice as fast as the corresponding bovine erythrocyte enzyme (compare rate constants at 26.0°C). The values of $k_r$ ($min^{-1}$) for the spontaneous recovery of the methylcarbamyl and dimethylcarbamyl bovine AChE at 26°C are 0.020 and 0.012, respectively, in good agreement with the values of 0.0234 and 0.0123 (25°C) reported by Reiner and Aldridge[1] for the same enzyme, although determined by a different procedure.

The magnitude of $E_a$ and $\Delta S^{\pm}$ for the regeneration of house fly and bovine AChE reveal distinct differences between the two enzymes. $E_a$ for the regeneration of carbamylated house fly AChE is substantially greater than that for bovine AChE, in spite of the faster rate of decarbamylation by the former. Evidently, the larger activation energy for house fly AChE is compensated by favorable entropy effects.

The activation energies for decarbamylation of house fly and bovine AChE are larger than the 14 kcal/mole observed for the alkaline hydrolysis of p-nitrophenyl methylcarbamate[6], but they approach those reported for the alkaline hydrolysis of ethyl methylcarbamate and dimethylcarbamate[7]. This is consistent with the general belief that carbamylation, acylation, and phosphorylation takes place on an aliphatic hydroxyl moiety in the active site, i.e., the serine hydroxyl.

Salt Effects - The primary and secondary salt effects on reaction rates have been well investigated. More recently Bruice et al.[8], have investigated the structure-forming properties of different salts on aqueous transition states. While the interpretation of primary or secondary salt effects could lend information regarding the mechanism of decarbamylation, pronounced salt effects were not found in this study. There was probably a secondary nonspecific salt effect which is consistent with acid-base catalysis, but this is to be expected.

Deuterium Oxide Effect - Deuterium oxide has been employed previously in the differentiation of general base and nucleophilic catalysis of hydrolysis. The values of kH/kD are generally less than 2.0 for nucleophilic catalysis due to the secondary isotope effect. Deuterium oxide was used as the sole solvent in the experiments cited in Table 2. Inhibitors 3-isopropylphenyl methylcarbamate (I) and 3-isopropyl-4-nitrophenyl dimethylcarbamate (II) were used to carbamylate bovine

Table 1. RATE CONSTANTS ($k_r$) AND ACTIVATION PARAMETERS FOR THE REGENERATION OF INHIBITED CHOLINESTERASE BY 3-ISOPROPYLPHENYL METHYLCARBAMATE AND 3-NITROPHEHYL DIMETHYLCARBAMATE AT pH 7.9

| Enzyme | Carbamate | T($^{o}$C) | $k_r$ (min$^{-1}$) | $E_a$ (Kcal/mole) | ΔS± (e.u.) |
|---|---|---|---|---|---|
| Fly | I 3-isopropylphenyl methylcarbamate | 22.0 | 0.0258 | 25.7 | 11.3 |
| | | 26.0 | 0.0465 | | |
| | | 29.5 | 0.104 | | |
| | | 32.5 | 0.105 | | |
| Fly | II 3-isopropyl-4-nitrophenyl dimethylcarbamate | 18.5 | 0.00735 | 27.9 | 17.2 |
| | | 22.0 | 0.0135 | | |
| | | 26.0 | 0.0225 | | |
| | | 29.5 | 0.0400 | | |
| | | 32.5 | 0.0707 | | |
| Bovine | I 3-isopropylphenyl methylcarbamate | 18.5 | 0.00539, 0.00583 | 19.8 | -2.8 |
| | | 22.0 | 0.00675, 0.00863 | | |
| | | 26.0 | 0.0200 | | |
| | | 33.0 | 0.0242 | | |
| Bovine | II 3-isopropyl-4-nitrophenyl dimethylcarbamate | 18.5 | 0.00394, 0.00450 | 16.4 | -3.5 |
| | | 26.0 | 0.0122 | | |
| | | 33.0 | 0.0142 | | |

Table 2. EFFECT OF DEUTERIUM OXIDE ON THE REGENERATION RATE OF CARBAMATE INHIBITED BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE AT pD of 8.42

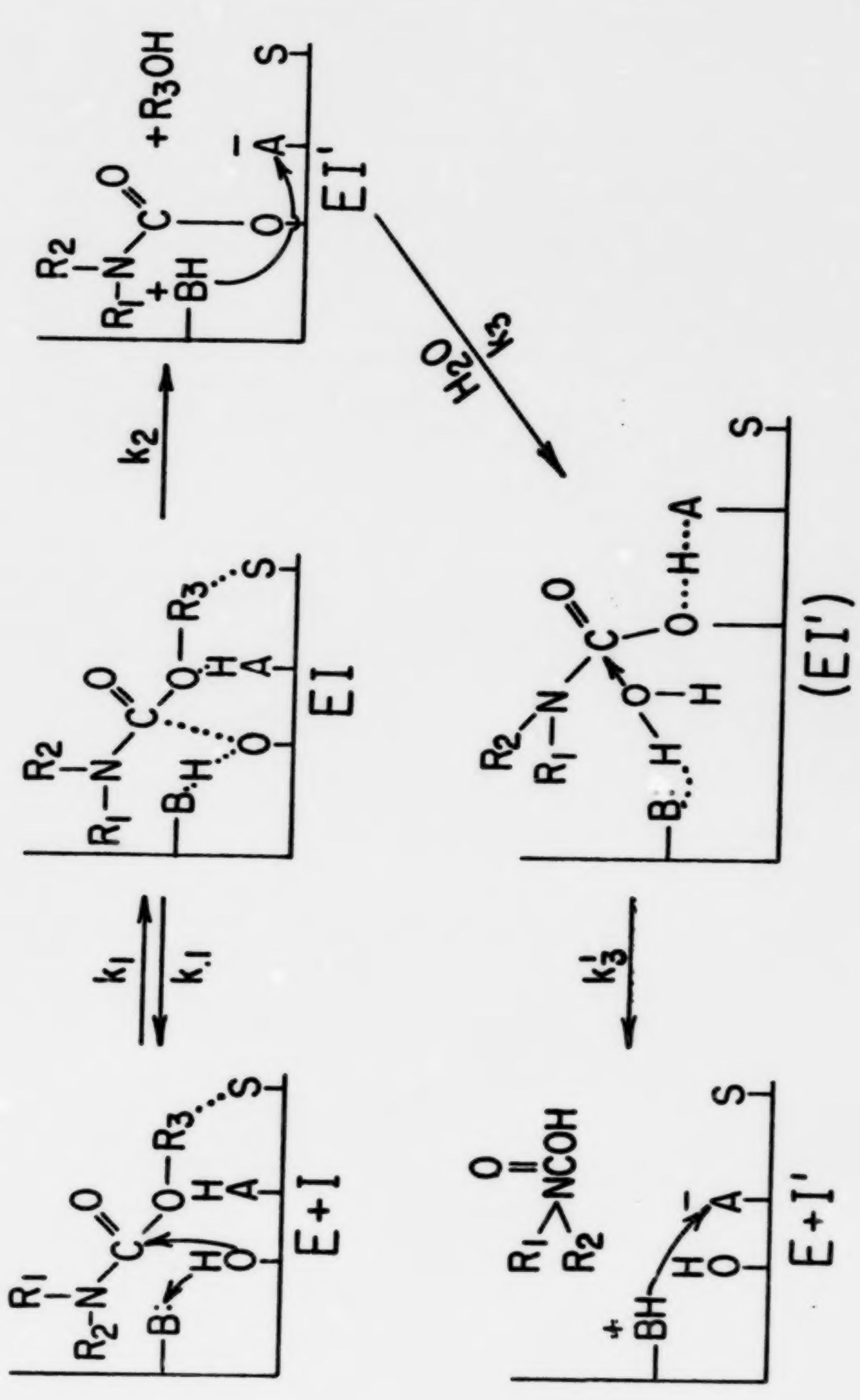
| Inhibitor | Solvent | $k_r$ | $k_h/k_d$ |
|---|---|---|---|
| 3-Isopropylphenyl methylcarbamate | water | 0.0508±0.0048 | |
| | deuterium oxide | 0.00674±0.00033 | 7.5 |
| 3-Isopropyl-4-nitrophenyl dimethylcarbamate | water | 0.0345±0.00070 | |
| | deuterium oxide | 0.0060±0.00046 | 5.7 |

erythrocyte AChE. The isotope effect of 5.7 for dimethylcarbamoyl enzyme regeneration and 7.5 for monomethylcarbamyl enzyme is consistent with a single proton transfer mechanism being operative in both cases and that general acid-base assisted nucleophilic attack by water is probably involved[9]. The magnitude and direction of the isotope effect supports this preliminary conclusion.

Conclusion - The method used in this study of the regeneration of AChE inhibited by methyl- and dimethylcarbamate esters differs from that normally used[1] in two basic respects. First, excess inhibitor (carbamate) was separated from the enzyme-carbamyl enzyme mixture by Sephadex chromatography. The general procedure used to minimize or terminate continuous carbamylation has been the "dilution" technique. Second, the rate of spontaneous regeneration of the carbamyl enzyme was monitored in the presence of the substrate ATCh by following the increase in the velocity of substrate hydrolysis with time. As expected, a positive curvature was obtained in the relationship between enzymatic activity and time. In the method normally used, the diluted enzyme-inhibitor mixture is allowed to stand and aliquot samples are taken for assay of enzymatic activity after fixed time intervals by addition of substrate (ACh or ATCh). This method requires that initial velocities for substrate hydrolysis be used to calculate the first-order regeneration constant since the velocity surely will increase with time as regeneration occurs.

The major difficulty in the present method resides in the proper analysis of the data. For this purpose, a mathematical model was established and regeneration constants were calculated by computer analysis. Another problem lies in the possibility that the reagents used to estimate substrate hydrolysis, i.e., ATCh, DTNB and its products, may have some effect on the regeneration rate. This may be especially true with respect to the substrate (ATCh) since it has been shown[10] that decarbamylation or methylcarbamylated AChE is inhibited by acetylcholine. However, in spite of these possible effects, the values obtained for $k_r$ by this method are similar to those reported by other investigators[1].

Models for the hydrolysis of ACh by AChE propose that both acetylation and deacetylation of AChE are mediated by acid-base catalysis. The mechanism of deacetylation is suggested as being the reverse of acetylation. The results from this study using $^{14}C$-labeled carbamates and of the effect of pH, deuterium oxide, and salts on the regeneration of carbamylated AChE suggest that a similar mechanism is in operation in the carbamylation and decarbamylation of AChE by carbamate esters. A mechanism for these processes consistent with that proposed for ACh hydrolysis is presented by the scheme below. Although the actual inhibition reaction was not examined in this study, the scheme includes the carbamylation process based on analogy with the model for ACh hydrolysis. In particular, the effect of pH and deuterium oxide


E+I
k1
k-1
EI
k2
+R3OH
EI'
H2O
k3
(EI')
k'3
E+I'
S
A
B

indicates that decarbamylation is acid-base catalyzed as depicted in the scheme.

The pH-rate profiles for decarbamylation for methyl- and dimethylcarbamyl AChE is compatible with a mechanism in which a water molecule attacks the carbonyl carbon of the carbamate moiety with assistance by the basic (imidazole) and acid (tyrosine OH) groups in the active site. This sort of bifunctional mechanism would not develop a formal charge and so a salt effect would be expected to be slight, if existent. The relatively small salt effect is perhaps consistent in this regard.

The deuterium oxide effect of 5.7-7.5 for $k_H/k_D$ is of the magnitude cited for a kinetic isotope effect which would be consistent with a proton transfer being rate limiting. The values of 6-10 $k_H/k_D$ in the reactions of normal proton transfer with nucleophiles has been cited as indicative of nucleophilic attack involving proton transfer. The deuterium oxide effect observed in this study is consistent with water being directly involved in decarbamylation as indicated in the scheme.

## 2. Aryl N-Hydroxy- and N-Methoxy-N-Methylcarbamates as Potent Reversible Inhibitors of Acetylcholinesterase

Introduction - In the preceding section a mechanism for the inhibition of AChE by carbamate esters and spontaneous regeneration of the inhibited enzyme was described. As indicated, the inhibition of AChE undoubtedly occurs by a carbamylation process in which an actual covalent bond is formed between the enzyme and inhibitor. In the course of our investigations on the effect of structural modification of the carbamoyl moiety of known carbamate insecticides on anticholinesterase and insecticidal activity, we have discovered a number of new aryl N-hydroxy- and N-methoxy-N-methylcarbamates which behave distinctly as reversible, competitive inhibitors of AChE. This section provides evidence that these carbamate derivatives inhibit AChE exclusively by a reversible competitive mechanism, i.e., by virtue of the complex EI as depicted in the first step of the scheme in part D-1.

Evidence for a Reversible, Competitive Mechanism for AChE Inhibition - Evidence supporting a reversible, competitive mechanism for the inhibition of bovine erythrocyte AChE by aryl N-hydroxy- and N-methoxy-N-methylcarbamates is given below for the m-isopropylphenyl methylcarbamate derivatives.

The degree of AChE inhibition by a fixed concentration of inhibitor was the same whether the enzyme was incubated with inhibitor for different time intervals before substrate was added or when the substrate and inhibitor were added simultaneously to the enzyme. Plots showing the rate of ATCh hydrolysis after inhibition with a fixed amount of m-isopropylphenyl N-hydroxy-N-methylcarbamate (V) are given in Fig. 5A. The parallel lines show that the activity of the enzyme was constant after different time intervals for the inhibition reaction and that equilibrium between enzyme, inhibitor, and substrate was established virtually instantaneously. As expected, residual enzymatic activity clearly was a function of the concentration of V (Fig. 5B). The characteristic time-independent and concentration-dependent behavior of AChE inhibition by V satisfied our first criterion that inhibition occurred in a reversible manner. The same phenomenon was observed with all other aryl N-hydroxy- or N-methoxy-N-methylcarbamates examined.

Incubation of enzyme and V for long periods of time (12 hr) had no effect on the degree of AChE inhibition but marked differences were noted when the enzyme was allowed to stand with m-isopropylphenyl N-methylcarbamate (VI), a typical carbamylating inhibitor (cf. Fig. 6). The inhibition profile obtained with VI shows an initial exponential decrease in enzymatic activity (to about 1 hr), eventually followed by a gradual increase in activity owing to regeneration of the enzyme by decarbamylation ($k_3$ step in scheme).

It is worth mentioning that N-hydroxypropoxur (XI in Table 3) has been reported as a carbamylating agent with spider mite acetylcholinesterase[11]. In this study the inhibitor (XI) and substrate were added simultaneously to the enzyme and the decrease in enzymatic activity was monitored with time. The author reported that the reaction between XI and spider mite AChE reached a steady-state situation unusually fast and decarbamylation occurred very rapidly, therefore, accurate kinetic data for $K_a$ and $k_2$ could not be obtained. However, the author failed to investigate the possibility that XI was behaving as a reversible inhibitor by pre-incubating the inhibitor and enzyme or examine long-term incubation effects on enzyme inhibition.

Gel filtration provided additional evidence that V and related esters are reversible AChE inhibitors. For example, 15-min incubation of AChE in a solution containing 5 X $10^{-4}$ M m-isopropylphenyl N-methoxy-N-methylcarbamate (VII) and analysis for enzymatic activity by adding ATCh (final concentration of VII was 3.3 X $10^{-6}$ M) showed that 56% of the enzyme was inhibited. However, analysis of the eluant obtained after passage of the original enzyme-inhibitor mixture through Sephadex resulted in virtually 100% recovery of enzymatic activity. On the other hand, AChE treated under the same conditions with $10^{-3}$ M m-isopropylphenyl N-(methoxy)-methyl-N-methylcarbamate (VIII), a carbamylating inhibitor, gave 7 and 15% enzyme activity before and after column filtration, respectively.

Finally, several of the N-hydroxy- and N-methoxy-derivatives were examined by the conventional kinetic methods to test for competitive inhibition. Figure 7 provides the double reciprocal plot for the inhibition of AChE by VII. The results clearly show that

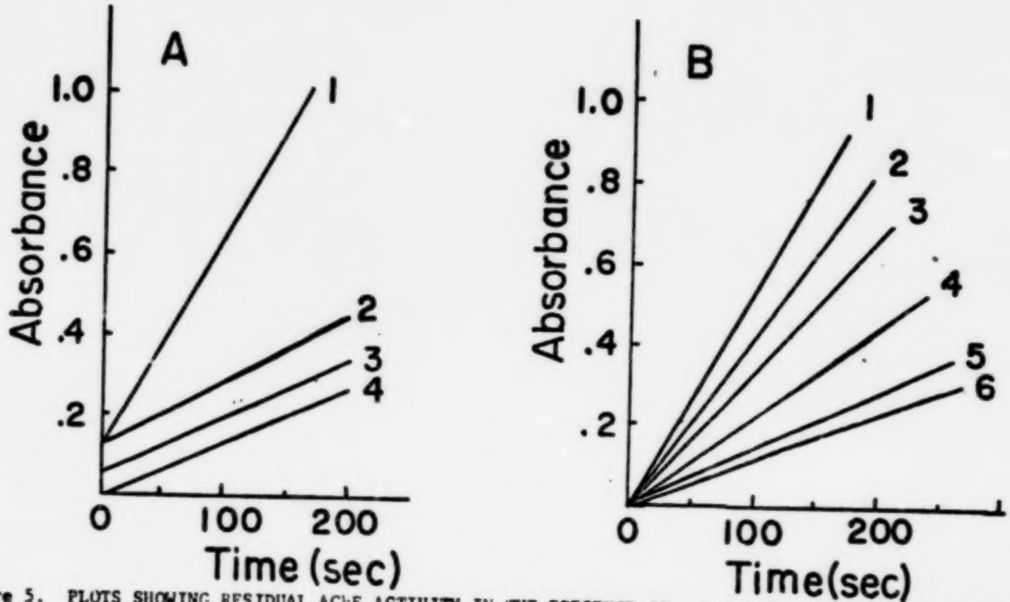


Figure 5. PLOTS SHOWING RESIDUAL AChE ACTIVITY IN THE PRESENCE OF *m*-ISOPROPYLPHENYL *N*-METHYLCARBAMATE (V). A. EFFECT OF TIME ON RESIDUAL ACTIVITY IN THE PRESENCE OF $1.33 \times 10^{-6}$ *M* V; (1) CONTROL ENZYME, (2) ENZYME ADDED TO MIXTURE OF SUBSTRATE AND INHIBITOR, (3) AND (4) PREINCUBATION WITH INHIBITOR FOR 10 AND 30 MIN, RESPECTIVELY. (PLOTS 1, 2 AND 3 WERE ARBITRARILY TRANSLOCATED FROM THE ORIGIN FOR BETTER ILLUSTRATION) B. EFFECT OF INHIBITOR CONCENTRATION ON RESIDUAL AChE ACTIVITY; (1) CONTROL ENZYME (2)-(6) $1.33 \times 10^{-7}$, $3.33 \times 10^{-7}$, $6.67 \times 10^{-7}$, $13.3 \times 10^{-7}$ AND $16.7 \times 10^{-7}$ *M* INHIBITOR, RESPECTIVELY

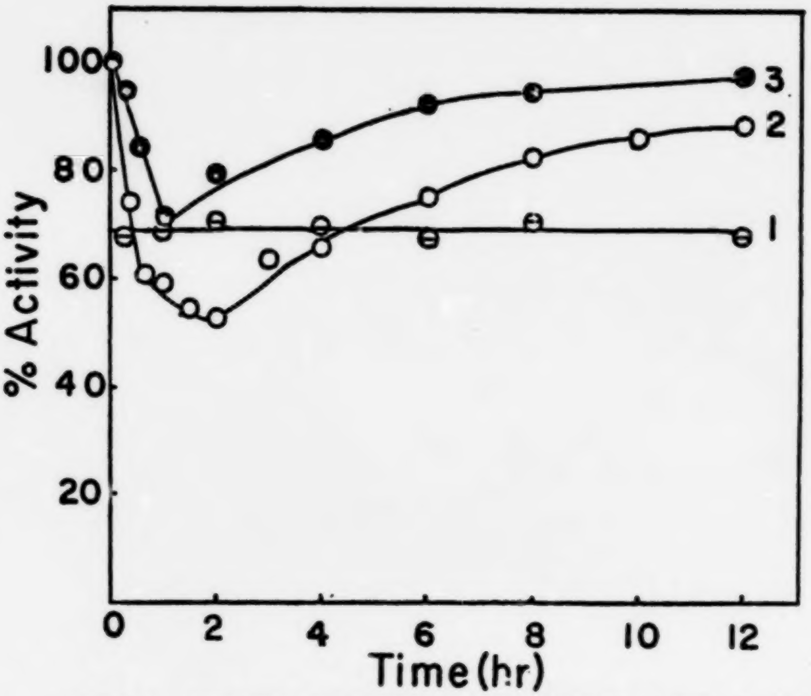


Figure 6. LONG-TERM TIME EFFECTS ON THE INHIBITION OF AChE BY *m*-ISOPROPYLPHENYL *N*-HYDROXY-*N*-METHYLCARBAMATE (V) AND *N*-METHYLCARBAMATE (VI), (1) AChE ACTIVITY IN THE PRESENCE OF 5 X $10^{-8}$ *M* V, (2) and (3) ACTIVITY IN THE PRESENCE OF 3 X 10 AND 1.5 X $10^{-8}$ *M* VI (PREINCUBATION CONCENTRATION), RESPECTIVELY

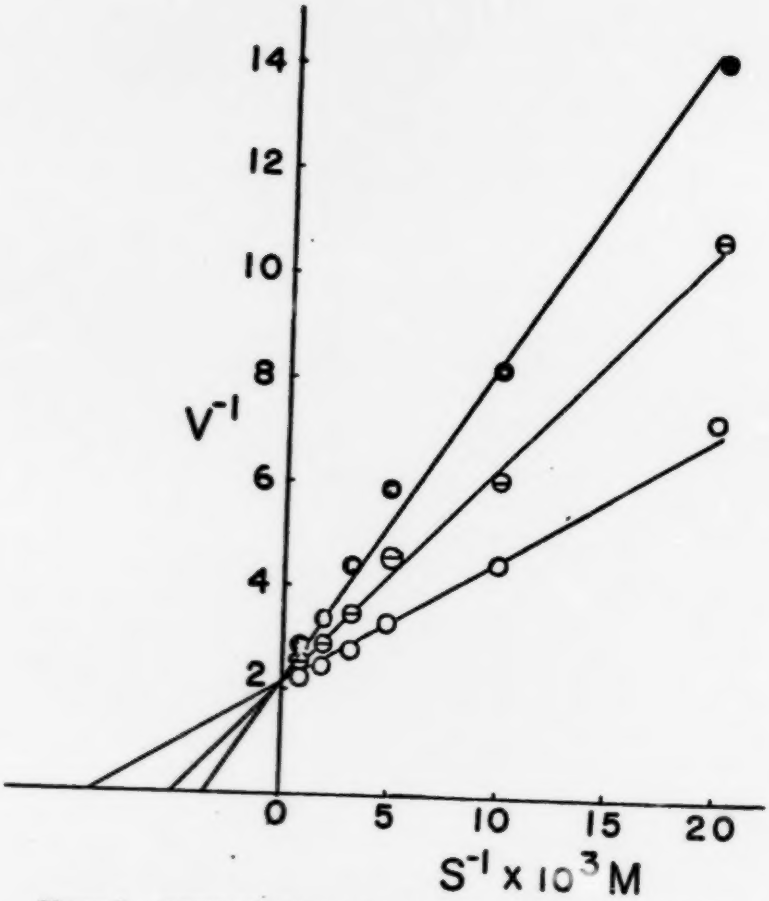


**Figure 7** DOUBLE-RECIPROCAL PLOT FOR THE INHIBITION OF AChE BY m-ISOPROPYLPHENYL N-METHOXY-METHYLCARBAMATE (VII), ○ CONTROL ENZYME, ⊖ $8.33 \times 10^{-7}$ M VII, ● $1.67 \times 10^{-6}$ M VII

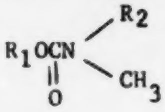
inhibition is reversible and competitive.

Values for $K_a$, or the dissociation constant for enzyme-inhibitor complex (EI), are given in Table 3 for the various N-hydroxy- and N-methoxy-N-methylcarbamates. $K_a$ in the case of reversible inhibitors is identical to $K_i$, the conventional expression for the reversible inhibition constant. As indicated in the table, the N-hydroxy- and N-methoxy-derivatives were unusually strong reversible inhibitors of AChE with $K_a$ ($K_i$) values in the range of $2 \times 10^{-5}$-$1 \times 10^{-7}$ M. Also, the N-hydroxy esters were generally more effective than the corresponding N-methoxy analogs. The data show that the affinity of these compounds for the enzyme is substantially greater than that previously reported for eserine and neostigmine, both of which are strong irreversible inhibitors of AChE[12].

Evidence also was obtained which showed that these reversible inhibitors are able to protect AChE from inhibition by irreversible inhibitors. This was demonstrated by examining the effect of V on the rate of inhibition of AChE by the potent anticholinesterase, Amiton (O,O-diethyl S-2-diethylaminoethylphosphorothioate oxalate salt), and by VI (Fig. 5). In the presence of $1 \times 10^{-7}$ M (V), the first-order rate of inhibition of AChE by $3 \times 10^{-7}$ M Amiton was reduced 3.3-fold, i.e., from 0.39 to 0.12 $\text{min}^{-1}$. Similarly, using $1 \times 10^{-7}$ M (V), the rate of inhibition of AChE by $1 \times 10^{-6}$ M (VI) was reduced approximately 5-fold, from 0.58 to 0.12 $\text{min}^{-1}$. Although IV, at a concentration approximately equal to its $K_i$ value, markedly reduces the rate which AChE is inactivated by (VI) or Amiton, it is not certain whether these compounds are competing with (I) for the same active site of the enzyme. The 3.3- and 5-fold reduction in rate constants for inhibition by Amiton and (VI) in the presence of $10^{-7}$ M (V) suggests that (V) also may protect AChE from irreversible inhibition by more than one mechanism.

Limited studies on the stability of N-methoxy-N-methylcarbamate esters to alkali have revealed that they are substantially less susceptible to hydrolysis than the corresponding methylcarbamate. For example, the second-order constant for hydroxide ion catalyzed hydrolysis of p-nitrophenyl N-methoxy-N-methylcarbamate is 4.5 $M^{-1}\ \text{min}^{-1}$ compared to $3.5 \times 10^{5}$ $M^{-1}\ \text{min}^{-1}$ for p-nitrophenyl methylcarbamate, a difference of approximately $7.6 \times 10^{4}$-fold. The N-methoxycarbamates evidently are quite stable and the low reactivity of the N-methoxycarbamate moiety to nucleophilic attack is consistent with the finding that these compounds evidently do not carbamylate AChE.

Table 3. DISSOCIATION CONSTANT ($K_a$) OF ARYL N-HYDROXY- AND N-METHOXY-N-METHYLCARBAMATES AND OTHER IRREVERSIBLE CARBAMATES



| | $R_1$ | $R_2$ | Type of inhibition | $K_a$ (M) |
|---|---|---|---|---|
| V | m-isopropylphenyl | OH | Reversible | $1.1 \times 10^{-7}$ |
| VI | m-isopropylphenyl | H | Irreversible | $1.8 \times 10^{-4}$ |
| VII | m-isopropylphenyl | $OCH_3$ | Reversible | $6.7 \times 10^{-7}$ |
| VIII | m-isopropylphenyl | $CH_2OCH_3$ | Irreversible | - |
| IX | m-trimethylammonium-phenyl | $OCH_3$ | Reversible | $1.6 \times 10^{-7}$ |
| X | m-dimethylaminophenyl | $OCH_3$ | Reversible | $1.9 \times 10^{-5}$ |
| XI | o-isopropoxyphenyl | OH | Reversible | $1.6 \times 10^{-7}$ |
| XII | o-isopropoxyphenyl | $OCH_3$ | Reversible | $9.5 \times 10^{-6}$ |
| XIII | (Eserine) | H | Irreversible | $3.3 \times 10^{-6}$ |
| XIV | m-trimethylammonium- | $CH_3$ | Irreversible | $1.2 \times 10^{-5}$ |

Conclusion - The results clearly show that the aryl N-hydroxy- and N-methoxy-N-methylcarbamates examined in this study are potent, reversible inhibitors of AChE with $K_i$ values as low as $1 \times 10^{-7}$ M. While none of these carbamate derivatives showed any insecticidal activity, they are of potential usefulness in basic studies on the nature of the active site in the cholinesterase enzymes. Indeed, they may be used in protecting the active site from phosphorylating and carbamylating agents. Since these carbamate derivatives are reversible inhibitors, the relative values of their affinity constant ($K_a$) may provide a direct measure of substituent effects on the binding of the carbamate to the enzyme. Aryl N-hydroxy- and N-methoxy-N-methylcarbamates, therefore, may be used to assess the effect of the nature and position of ring substituents on carbamate binding to AChE without complication from the carbamylation reaction. Further work along these lines is in progress.

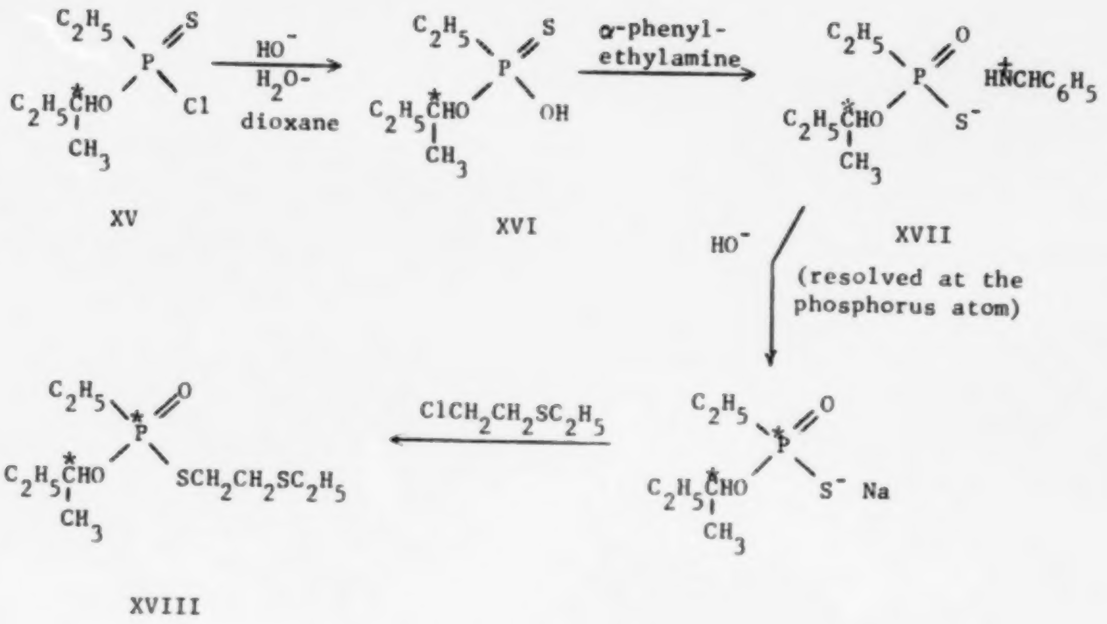
## 3. Stereoselectivity in Cholinesterase Inhibition

Introduction - Stereoselectivity in the interaction of molecules containing asymmetric centers with biological systems is a well known phenomenon, and the interaction of asymmetric substrates and inhibitors with cholinesterase is no exception. This section is concerned with the synthesis, resolution, and examination of the toxicological properties of organophosphorus esters containing two asymmetric centers. In particular, work was conducted to determine the effect of simultaneous chirality at both the phosphorus atom and the α-carbon atom of the alkoxy moiety in phosphonothioate esters on the inhibition of cholinesterase from three different sources. Additional experiments were performed to determine whether these effects were reflected in the toxicity of the chiral organophosphorus esters to insects and mice and in their plant systemic insecticidal effectiveness.

Synthesis and Resolution - The following reactions were used for the preparation and resolution of the organophosphorus compounds used in this study. D- and L-isomers of 2-butanol were purchased from Norse Laboratories, Santa Barbara, CA. Chiral centers are indicated by asterisks. The α-phenylethylamine salt (XVII) was used for resolution of the 4 diastereomer salts which were subsequently converted to the resolved esters of XVIII by treatment with 2-chlorodiethyl sulfide. The absolute configuration of the two asymmetric centers (C and P) in XVIII and the magnitude of rotations are given in Table 4.

Table 4. OPTICAL ROTATION IN DEGREES OF ARC AND ABSOLUTE CONFIGURATION OF THE VARIOUS ISOMERS OF XVIII

| Isomer | Configuration at<br>C | <br>P | $\alpha^{26}_D$ |
|---|---|---|---|
| XVIIIa | S | S | -33.371 |
| XVIIIb | S | racemic | +11.072 |
| XVIIIc | S | R | +60.098 |
| XVIIId | R | S | -60.276 |
| XVIIIe | R | racemic | -14.698 |
| XVIIIf | R | R | +33.780 |



Totally racemic XVIII was treated with m-chloroperbenzoic acid to give the corresponding sulfoxide (XIX) and with potassium permanganate to give the sulfone (XX).

Cholinesterase Inhibition - Bimolecular inhibition constants ($k_i$) were determined for cholinesterase from three different sources. Anticholinesterase data ($k_i$) for the various isomers of XVIII against house fly head (HFAChE), bovine erythrocyte (BAChE), and horse serum (HSChE) cholinesterases are presented in Table 5. Wide variability was observed in the inhibitory behavior of the various isomers to each enzyme and in the behavior of each isomer to the different enzymes. For example, the enantiomers XVIIIc and XVIIId gave inhibition constants of $1.35 \times 10^3\ \underline{M}^{-1}\ \text{min}^{-1}$ and $1.69 \times 10^6\ \underline{M}^{-1}\ \text{min}^{-1}$, respectively, against HFAChE, a rate difference of about 1250-fold. Similar and consistent, though smaller, rate differences were observed between the enantiomers of XVIII in their inhibition of the other two cholinesterases. The largest rate differences were caused by chirality at the phosphorus atom. The (S)-phosphorus isomers consistently gave larger inhibition constants than the corresponding (R)-phosphorus isomers; i.e., compare XVIIIa with XVIIIc and XVIIId with XVIIIf.

The effect of chirality at the α-carbon (2-butyl) on cholinesterase inhibition was dependent upon chirality at the phosphorus atom. In the two isomers with the (S)-phosphorus atom, the (S)-α-carbon compound (XVIIIa) gave a larger $k_i$ than the (R)-α-carbon compound (XVIIId). However, in the

Table 5. ANTICHOLINESTERASE PROPERTIES OF XVIII, ITS RESOLVED ISOMERS, XIX, AND XX AGAINST HOUSE FLY-HEAD ACETYLCHOLINESTERASE (HFAChE), BOVINE ERYTHROCYTE ACETYLCHOLINESTERASE (BAChE), AND HORSE SERUM CHOLINESTERASE (HSChE)

|  | Config. |  | HFAChE $k_i$ (95% CI) | BAChE $k_i$ (95% CI) | HSChE $k_i$ (95% CI) |
|---|---|---|---|---|---|
| No. | C | P | $M^{-1}$ $min^{-1}$ X $10^{-3}$ | $M^{-1}$ $min^{-1}$ X $10^{-3}$ | $M^{-1}$ $min^{-1}$ X $10^{-3}$ |
| XVIII | racemic |  | 969<br>(29) | 31.8<br>(0.9) | 5.95<br>(0.21) |
| XVIIIa | S | S | 1710<br>(50) | 65.3<br>(2.1) | 17.3<br>(0.9) |
| XVIIIc | S | R | 1.35<br>(0.02) | 0.629<br>(0.020) | 0.483<br>(0.042) |
| XVIIId | R | S | 1690<br>(110) | 54.5<br>(2.2) | 6.85<br>(0.30) |
| XVIIIf | R | R | 6.62<br>(0.30 | 1.45<br>(0.07) | 1.01<br>(0.18) |
| XIX | racemic |  | 556<br>(21) | 43.9<br>(1.4) | 3.67<br>(0.17) |
| XX | racemic |  | 696<br>(39) | 126<br>(8) | 15.1<br>(0.6) |

other two isomers with the (R)-phosphorus atom, the (R)-$\alpha$-carbon compound (XVIIIf) gave a larger $k_i$ than the (S)-$\alpha$-carbon compound (XVIIIc). Thus, in all three cholinesterases, the order of rate constants ($k_i$) for the four fully resolved isomers of XVIII is XVIIIa > XVIIId >> XVIIIf > XVIIIc. The consistency of these results indicates that these asymmetric inhibitors are interacting with the active site of each enzyme in a similar manner and, therefore, the stereochemistry involved at the active site of the three enzymes is probably similar.

Several additional points of interest emerge from the $k_i$ data in Table 5. It can be seen that among the compounds listed, HFAChE is more sensitive to inhibition than BAChE, which in turn is more sensitive than HSChE. Comparing, for example, the relative rates of inhibition of the three enzymes by XVIIIa, HFAChE is 26-fold more sensitive to inhibition than BAChE which, in turn, is 3.8-fold more sensitive than HSChE. It can also be seen that the largest rate differences are produced by the more effective levarotatory (S)-phosphorus inhibitors (XVIIIa, XVIIId). The dextrorotatory (R)-phosphorus compounds (XVIIIc, XVIIIf) showed rather low and approximately equal activity against the three enzymes, although XVIIIf was moderately active against HFAChE. In contrast, anticholinesterase activity increased dramatically for the (S)-phosphorus isomers (XVIIIa, XVIIId) in proceeding from HSChE to BAChE to HFAChE.

As expected, $k_i$ for racemic XVIII against each of the three cholinesterases closely equalled the weighted sum of $k_i$ values of the individual resolved inhibitors (XVIIIa, XVIIIc, XVIIId, XVIIIf) after adjustment for concentration. Against BAChE, the weighted sum of the $k_i$ values for the four resolved isomers is $3.06 \times 10^4$ $\underline{M}^{-1}$ min$^{-1}$, compared to an observed $k_i$ of $3.18 \times 10^4$ $\underline{M}^{-1}$ min-1 for XVIII. These results indicate that the individual isomers in the racemic mixture are acting independently of each other.

Against all of the enzymes used, $k_i$ for the sulfone (XX) was larger than that for the sulfoxide (XIX) probably because of the greater reactivity of XX. Against BAChE and HSChE, $k_i$ of XIX was larger than that for XVIII; however, against HFAChE, $k_i$ for both XIX and XX was smaller than that for XVIII.

Toxicity - In general, toxicity to either insects or to mice paralleled anticholinesterase activity, i.e., the stronger inhibitors proved to be more effective toxicants to each test animal (Table 6). The (R)-phosphorus isomers (XVIIIc,f) were nontoxic at the maximum dosages used to all of the insects tested and only moderately toxic to mice. The (S)-phosphorus isomers (XVIIIa,d) were highly toxic to susceptible house flies and mice and moderately toxic to resistant house flies and mosquito larvae. Small differences in toxicity owing to chirality at the 2-butyl carbon atom also were noted.

Table 6. TOXICITY OF XVIII, ITS ISOMERS, XIX AND XX, TO HOUSE FLIES (TOPICAL, ADULT, FEMALE), MOSQUITO LARVAE AND WHITE MICE (ORAL)

| No. | *Musca domestica* NAIDM ($LD_{50}$ µg/g) | SC ($LD_{50}$ µg/g) | Larvae ($LC_{50}$ ppm) | Mouse ♀ ($LD_{50}$ µg/g) |
|---|---|---|---|---|
| XVIII | 10.8 | 78 | 0.65 | 3.1 |
| XVIIIa | 6.7 | 33 | 0.25 | 3.2 |
| XVIIIb | 9.4 | 84 | 0.66 | - |
| XVIIIc | >500 | >500 | >1 | 110 |
| XVIIId | 6.9 | 42 | 0.15 | 2.8 |
| XVIIIe | 12.1 | 122 | 0.57 | - |
| XVIIIf | >500 | >500 | >1 | 125 |
| XIX | 39 | 310 | >1 | - |
| XX | 27 | >500 | >1 | - |

Because the (R)-phosphorus isomers were nontoxic to insects at the maximum dosages used, it is not possible to determine whether a direct correlation exists between in vitro anticholinesterase activity of the four isomers of XVIII and insecticidal activity. For example, against HFAChE, $k_i$ ($\underline{M}^{-1}$ min$^{-1}$) for XVIIIa and XVIIIc are 1.71 X $10^6$ and 1.35 X $10^3$, respectively, a difference of about 1270-fold. In comparison, the $LD_{50}$ values (μg/g) of XVIIIa and XVIIIc to house flies are 6.7 and >500, respectively, a difference of >75-fold.

A similar comparison may be made between inhibition of BAChE and mouse toxicity. Owing to the appreciable toxicity of the (R)-phosphorus enantiomers, calculation of finite values for the ratio of toxicities was possible. Against BAChE, XVIIIa ($k_i$ 6.53 X $10^4$ $\underline{M}^{-1}$ min$^{-1}$) was 104-fold more effective as an inhibitor than XVIIIc ($k_i$ 6.29 X $10^2$ $\underline{M}^{-1}$ min$^{-1}$) and 34-fold more toxic to the white mouse (3.2 and 110 mg/kg, respectively). Similarly, XVIIId was 45-fold more effective as an anticholinesterase than XVIIIf and 37-fold more toxic to the white mouse. Considering that biological systems are involved, the relationship between inhibition of a mammalian acetylcholinesterase, BAChE, and toxicity to the white mouse is quite good and suggests that other factors involved in intoxication (metabolism, translocation, etc.) are similar for the different isomers in the mouse.

Plant Systemic Activity - Data for plant systemic activity in cotton plants against 4 pest species are presented in Table 7. Indicated are the number of weeks in which >50% mortality of the test species were obtained following granular treatment of the soil or topical stem application of young cotton plants. The longer the mortality is sustained at a high level, the better the toxicant is as a plant systemic insecticide. Similar data for aldicarb, a well-known plant systemic insecticide, are included for comparison.

The dextrorotatory (R)-phosphorus isomers (XVIIIc,f) were completely inactive as plant systemic insecticides. It is not certain whether the absence of activity is attributable to decreased rates of absorption, translocation, and perhaps metabolism in the plant owing to stereoisomerism or whether the translocated isomers (or their metabolites) themselves are nontoxic owing to their poor anticholinesterase properties. In contrast, the levorotatory (S)-phosphorus isomers (XVIIIa,d) were highly active, a finding which is consistent with their high anticholinesterase activity toward HFAChE. Since it is unlikely that the physical properties of the individual isomers which affect movement in plants vary to any large extent, the poor systemic activity of XVIIIc and XVIIIf probably is attributable to poor anticholinesterase activity.

Table 7. PLANT SYSTEMIC ACTIVITY, THE NUMBER OF WEEKS WITH A 50 PER CENT MORTALITY OF THE TEST ARTHROPODS. THE ARTHROPODS ARE *TETRANYCHUS CINNABARINUS* (MITE), *APHIS GOSSYPII* (APHID), *BUCCULATRIX THURBERIELLA* (PERFORATOR), AND *ESTIGMENE ACREA* (SALT MARSH). 4 = 400 mg SOIL TREATMENT, 8 = 800 mg SOIL TREATMENT, S = STEM TREATMENT

| Compound | Treatment | Mite | Aphid | Perforator | Salt Marsh |
|---|---|---|---|---|---|
| XVIII | 4 | 4 | 4 | 4 | 0 |
| | 8 | 4 | 5 | 4 | 3 |
| | S | 4 | 4 | 4 | 1 |
| XVIIIa | 4 | 12[a] | 15 | 12 | 2[b] |
| | 8 | 12 | 16 | 12 | 4[a] |
| | S[c] | 3 | 3 | 3 | 2 |
| XVIIIc | 4,8,S | All completely inactive | | | |
| XVIIId | 4 | 14[a] | 15 | 12[a] | 1 |
| | 8 | 14 | 15 | 12 | 1 |
| | S | 3 | 5[a] | 2 | 1 |
| XVIIIf | 4,8,S | All completely inactive | | | |
| Aldicarb | 4 | 10 | 14 | 15 | 3 |
| | 8 | 10 | 15 | 16 | 4 |
| | S | 3 | 7 | 14 | 0 |

[a] Variable, but with at least half of the points 90 per cent plus.

[b] Maximum to only 60 percent mortality.

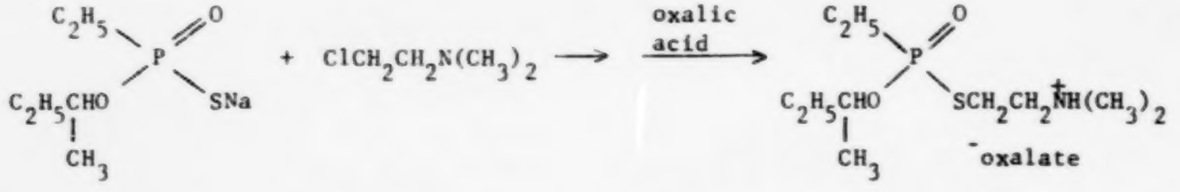
[c] Phytotoxic, plant dead in 3 weeks.

[d] No mortality for first 3 weeks.

[e] Average mortality about 40 per cent for some 14 weeks.

Little difference in the duration of activity between the two different dosages of 800 and 400 mg was found for any of the compounds, although the activity of the 800-mg treatment occasionally lasted an additional week. The activities of XVIIIa and XVIIId, the (S)-phosphorus isomers, are similar, with XVIIIa slightly more effective against salt marsh caterpillars and XVIIId more effective against mites. The striking observation here is that persistence of systemic activity following treatment with XVIIIa or XVIIIc was approximately three times longer than after treatment with racemic XVIII at 800 mg, even though the combined amount of XVIIIa and XVIIIc in XVIII at the higher dosage is equal to 400 mg. As soil insecticides, XVIIIa and XVIIId are superior to aldicarb against mites and aphids in these tests but slightly inferior to aldicarb against perforators and salt marsh caterpillars.

Affinity and Phosphonylation Constants - Studies on the behavior of organophosphorus esters containing two asymmetric centers were extended to the isomers of O-2-butyl S-2-(dimethylammonium)ethyl ethylphosphonothioate hydrogen oxalate (XXI). The various isomers were prepared in a manner analogous to the preparation of XVIII except O-2-butyl ethylphosphonothioic acid was reacted with 2-(dimethylamino)-chloroethane according to the equation below.



XXI

The absolute configuration of the C and P atoms of the individual isomers and magnitude of rotations are given in Table 8.

The enzymes studied were bovine erythrocyte (BAChE) and house fly-head (HFAChE) acetylcholinesterase, and horse serum pseudocholinesterase (HSChE). The kinetic treatment of the inhibition reaction was similar to that of Main and Iverson[13] based on the following inhibition scheme.

$$\begin{tikzcd}
E + I \arrow[r, shift left, "k_1"] \arrow[rr, bend right, "k_i"'] & (EI)^R \arrow[l, shift left, "k_{-1}"] \arrow[r, "k_p"] & (EI)^I
\end{tikzcd}$$

## Table 8. OPTICAL ROTATION IN DEGREES ARC AND ABSOLUTE CONFIGURATION OF RACEMIC (XXI) AND RESOLVED (XXIa-d) COMPOUNDS

| No. | Configuration | | $D^{26}$[a] |
|---|---|---|---|
| | C | P | (° of arc) |
| XXI | racemic | | - |
| XXIa | S(+) | S(-) | - 9.86 |
| XXIb | S(+) | R(+) | +48.96 |
| XXIc | R(-) | S(-) | -49.36 |
| XXId | R(-) | R(+) | +10.12 |

---

[a] The concentration was approximately 0.01 g/ml in absolute ethanol. The standard deviations of the observed rotations averaged 0.005°.

Free enzyme is E, I is inhibitor, $(EI)^R$ is a reversible enzyme-inhibitor complex, and $(EI)^I$ is irreversibly phosphorylated enzyme. The individual rate constants are $k_1$, $k_{-1}$, and the phosphorylation rate constant, $k_p$. The dissociation or affinity equilibrium constant, $K_a$, is equal to $k_{-1}/k_1$ and the bimolecular inhibition constant, $k_i$, is equal to $k_p/K_a$. The inhibition constants $K_a$, $k_p$, and $k_i$, can be evaluated by using the equation below[13], where i is inhibitor concentration and $\Delta t/\Delta \ln V$ is the reciprocal of the pseudo first-order rate of enzyme inhibition at inhibitor concentration i.

$$\frac{i\Delta t}{\Delta \ln V} = \frac{i}{k_p} + \frac{1}{k_i}$$

The kinetic constants, $K_a$, $k_p$, and $k_i$, for the inhibition of the three cholinesterases by racemic XXI and its four optical isomers (XVIa-d) are presented in Table 9. Also included are the ranges of inhibitor concentrations and the number of individual points used in each final weighted regression.

In four cases, i.e., in the inhibition of BAChE and HFAChE by XXI or XXIc, a nonlinear relationship was obtained in plots of $i\Delta t/\Delta \ln V$ against i. These plots typically showed an apparent upward curvature when the regression line approached the vertical axis. These points were discarded in the final regressions. A similar upward curvature has been reported by Main[14] for the inhibition of HSChE by amiton [O,O-diethyl S-2-(diethylamino)ethyl phosphorothioate] and by Iverson[15] for dimethylcarbamoyl choline inhibition of electric eel acetylcholinesterase in the presence of tetramethylammonium ion.

The data in Table 9 show that large variations exist in the bimolecular inhibition constants ($k_i$) determined for the various isomers for each enzyme and in the inhibitory activity of each isomer to the various enzymes. For example, against HFAChE, $k_i$ for XXId is $1.60 \times 10^3$ $M^{-1}$ $min^{-1}$ and that of its enantiomer (XXIa) is $1.65 \times 10^7$ $M^{-1}$ $min^{-1}$, a rate difference of 10,300-fold. Similar, though smaller, rate differences were observed between phosphorus enantiomers against the other cholinesterases. The magnitude of the $k_i$ values for each isomer against all of the enzymes, with the exception of XXIa and XXIb against HSChE follows the order XXIa > XXIc >> XXIb > XXId. The S-phosphorus isomers gave larger rate constants than the R-phosphorus isomers and, within each phosphorus enantiomer, the S-secondary butyl isomers gave slightly larger rate constants than the $R_C$-isomers. Against BAChE and HFAChE the R-phosphorus inhibitors gave rather small and approximately equal rate constants, e.g., the $k_i$ values for XXId against BAChE and HFAChE are $1.74 \times 10^3$ $M^{-1}$ $min^{-1}$ and $1.60 \times 10^3$ $M^{-1}$ $min^{-1}$, respectively. The same compound (XXId) gave a 13-fold larger $k_i$ value ($2.15 \times 10^4$ $M^{-1}$

$min^{-1}$) against HSChE than against HFAChE. In contrast, the house fly enzyme was considerably more sensitive to inhibition by the S-phosphorus isomers than was the bovine enzyme, e.g., the $k_i$ values for XXIc against HFAChE and BAChE are $1.46 \times 10^7$ $M^{-1}$ $min^{-1}$ and $2.25 \times 10^6$ $M^{-1}$ $min^{-1}$, respectively, a difference of 6.5-fold. HSChE was even less sensitive to inhibition by XXIc ($k_i = 8.63 \times 10^5$ $M^{-1}$ $min^{-1}$) than was the bovine enzyme. Overall rate differences ($k_i$ for XXIc vs $k_i$ for XXId) within each cholinesterase are 9120-fold for HFAChE, 1630-fold for BAChE and only 40-fold for HSChE. Thus, it appears that differences in anticholinesterase activity attributable to chirality at the phosphorus atom are much greater with acetylcholinesterase (BAChE and HFAChE) than with pseudo cholinesterase (HSChE).

The $k_i$ values for the inhibition of BAChE and HFAChE by racemic XXI closely equal the weighted sums of the $k_i$ values of the individual isomers. Against BAChE the weighted sum of the $k_i$ values for the four resolved isomers is $3.03 \times 10^6$ $M^{-1}$ $min^{-1}$, compared to an observed value of $3.50 \times 10^6$ $M^{-1}$ $min^{-1}$ for XXI. These results indicate that the individual isomers in the racemic mixture are inhibiting acetylcholinesterase independently of each other.

The rather large differences in $k_i$ between phosphorus enantiomers against HFAChE and BAChE are attributable mainly to $K_a$, the differences in affinity averaging around 220 fold. However, corresponding differences in $k_p$, averaging around 15 fold, also contributed. For example, comparison of XXIa and XXId for the inhibition of HFAChE shows that their respective affinity constants ($K_a$) are $6.70 \times 10^{-6}$ M and $1.95 \times 10^{-3}$ M, a difference of 290 fold, and their respective phosphonylation constants ($k_p$) are 111 $min^{-1}$ and 3.13 $min^{-1}$, a difference of 35 fold. These differences in affinity and phosphonylation constants combine to give a difference in $k_i$ of 10,340 fold. Further, against BAChE $K_a$ for XXIa and XXId is, respectively, $9.44 \times 10^{-6}$ M and $2.98 \times 10^{-3}$ M, a difference of 306 fold, and $k_p$ is 87.4 $min^{-1}$ and 5.02 $min^{-1}$, a difference of 17 fold. Combination of the affinity and phosphonylation constants yields a difference in $k_i$ of 5,330 fold. Compared to BAChE, the approximately 2-fold larger values for $k_i$ for the inhibition of HFAChE by the S-phosphorus isomers (XXIa, XXIc) is mainly attributable to larger phosphonylation constants.

Analogous comparisons with the horse serum enzyme, such as those made above between the fly and bovine enzymes, are complicated by the apparent separation of HSChE into different kinetic forms upon inhibition by compounds XXI, XXIa, and XXIb. A comparison of the results for XXIc and XXId, inhibitors toward which HSChE behaved as one kinetic form, reveals a pattern similar to that found in the acetylcholinesterases, but to a smaller degree. The difference of 40-fold in $k_i$ between XXIc and XXId against HSChE is attributable mainly to the 16.4-fold difference in $K_a$. The greater anticholinesterase activity ($k_i$) observed for the R-phosphorus isomer (XXId) against HSChE, compared to BAChE and HFAChE, is attributable to affinity since their respective phosphonylation constants ($k_p$) are rather similar. On the other hand, the lower anticholinesterase activity of the S-phosphorus isomer (XXIc) against HSChE, compared to the acetylcholinesterases, is attributable mainly to differences in $k_p$.

The effect of asymmetry at the 2-butyl carbon atom on anticholinesterase activity is small and variable. Comparison of XXIb and XXId against HFAChE shows that both $K_a$ and $k_p$ contribute equally to the 4-fold difference in $k_i$. For the same compounds against BAChE, the 4-fold difference in $k_i$ is attributable mainly to the 3-fold difference in $k_p$. For HSChE, the observed rate differences attributable to asymmetry at carbon cannot be determined accurately owing to the separation of the enzyme into different kinetic forms. Overall, the differences in kinetic parameters observed between carbon isomers are in agreement with those reported by Chiu and Dauterman[16] for the inhibition of BAChE by the enantiomers of malaoxon, where asymmetry resides on the $\alpha$-carbon of the diethyl mercaptosuccinate moiety. In this case, a 4-fold difference in $k_i$ was observed, attributable to 2-fold differences in both $K_a$ and $k_p$.

Based on pseudo first-order plots, the inhibition of HSChE by XXI, XXIa, and XXIb presents a special case in that the compounds appeared to separate the enzyme into different kinetic forms, designated as A, B, and C. It is apparent from the constants in Table 9 that the differences in the rates in which these kinetic forms are inhibited are primarily attributable to differences in phosphonylation constants, with the faster forms having larger phosphonylation constants than the slower forms. The magnitude of the differences in $k_i$ for the inhibition of the kinetic forms by XXI and XXIa, however, is reduced to some extent by the $K_a$ values. For example, even though $k_p$ for the inhibition of A by XXIa is 105-fold larger than for C, the 13-fold larger value of $K_a$ reduces the difference in $k_i$ between A and C to about 8 fold. The $K_a$ values for the inhibition of A, B, and C by XXIb are approximately the same and, therefore, $k_i$ for each kinetic form is directly proportional to the $k_p$ values. Racemic XXI separated the enzyme into two forms instead of three.

Comparable results were reported by Main[14] who also observed that the relative magnitude of the bimolecular inhibition constants for inhibition of the various kinetic forms of HSChE were controlled primarily by $k_p$. In addition, the horse serum enzyme was separated into four kinetic forms at 5°C, and only three forms with a possibility of a fourth at 25°C[14]. This agrees with the three forms found for XXIa and XXIb in this study at 30°C.

Although the various kinetic forms of HSChE are designated as A, B, and C in order of descending $k_i$ values, this is not to imply that the forms identified by a particular symbol are identical to each other for all three compounds (XXI, XXIa, or XXIb). The symbols are used merely to distinguish between the kinetic forms separable by each compound and it is possible that a form identified by one symbol for a given compound is not the same as a form identified by the same symbol for another compound. From the estimated values for the relative amounts of each form identified for XXIa and XXIb (cf Table 9), it appears that form A for XXIa (43%) is the same as form C for XXIb (41%). This may help to account for the rather anomalous division of HSChE into only two kinetic forms by XXI. XXI is an equimolar mixture of all four chiral isomers used and, therefore, eight independent reactions between the various isomers of XXI and the enzyme are expected, two of the isomers (XXIa and XXIb) reacting at different rates with the three kinetic forms and two isomers (XXIc and XXId) reacting at their respective rates with the enzyme as a whole. In the early stages of the reaction between XXI and HSChE, the rate which was observed (XXI-form A) is probably attributable primarily to the reaction between XXIa and its kinetic form A, since $k_i$ for this pair of reactants is substantially larger than that for any other pair of reactants. The fraction of the total enzyme that is included in XXI-form A (61%) is larger than the fraction in XXIa-form A (43%) because although the early portion of the reaction between XXI and HSChE is probably dominated by XXIa-form A, it is a sum of 4 linear additive reactions (XXIa-form A, XXIb-form C, and XXIc and XXId against the total enzyme). The remaining portion of the enzyme (XXI-form B) represents a sum of 6 reactions, (XXIa-forms B and C, XXIb-forms A and B, and XXIc and XXId against the total remaining enzyme). Probably XXI-form B shows linear first-order behavior because it is a sum of several inhibition reactions.

Reproducibility of results obtained by the spectrophotometric technique used, and the significance of the error terms, is illustrated by the three independent replicates of the inhibition of BAChE by the $R_CS_P$ isomer (XXIc). Two gave inhibition constants that were very similar to each other with small standard errors. The third replicate, covering a smaller concentration range, deviated slightly from the other two and had much larger standard errors.

A minimum estimate of the optical purity of these resolved inhibitors may be obtained by comparison of overall rate differences. The $S_CS_P$ inhibitor (XXIa) inactivates the house fly enzyme approximately 10,300-fold faster than the $R_CR_P$ compound (XXId). If the $R_P$ isomers (XXIb, XXId) are assumed to be completely inactive as inhibitors of cholinesterase, then any inhibitory activity observed in experiments using $R_P$ isomers should be caused by small contaminations by the $S_P$ isomers (XXIa, XXIc). From the observed rate difference of 10,300-fold, calculations indicate that there is less than 0.01 per cent contamination of $S_P$ isomers in the $R_P$ preparations.

*Conclusion* - Chirality in the organophosphorus ester *O*-2-butyl *S*-2-(ethylthio)ethyl ethylphosphonothioate evidently has a profound effect

Table 9. AFFINITY, EQUILIBRIUM, PHOSPHORYLATION RATE, AND BIMOLECULAR RATE CONSTANTS FOR THE INHIBITION OF THREE CHOLINESTERASES BY RACEMIC (XXI) AND ITS OPTICAL ISOMERS AT 30°C, pH 7.55

| No. | Config. | Form | % of Total enzyme | $K_a$ (SE) ($\underline{M}$ X $10^5$) | $k_p$ (SE) ($\text{min}^{-1}$) | $k_i$ ($\underline{M}^{-1}$ $\text{min}^{-1}$ X $10^{-5}$) | I ($\underline{M}$ range) | No. of points |
|---|---|---|---|---|---|---|---|---|
| | | | | Bovine Erythrocyte Acetylcholinesterase | | | | |
| XXI | racemic | | | 2.57 (0.37) | 89.9 (9.9) | 35.0 | 1 X $10^{-6}$-1 X $10^{-5}$ | 6 |
| XXIa | $\underline{S}_C\underline{S}_P$ | | | 0.944 (0.065 | 87.4 (4.0) | 92.6 | 1 X $10^{-7}$-1 X $10^{-6}$ | 9 |
| XXIb | $\underline{S}_C\underline{R}_P$ | | | 234 (25) | 15.5 (0.5) | 0.0648 | 5 X $10^{-4}$-1 X $10^{-2}$ | 8 |
| XXIc | $\underline{R}_C\underline{S}_P$ | | | 2.00 (0.13) | 56.7 (2.6) | 28.4 | 1 X $10^{-6}$-1 X $10^{-5}$ | 7 |
| XXIc | $\underline{R}_C\underline{S}_P$ | | | 1.98 (0.07) | 55.8 (1.4) | 28.2 | 1 X $10^{-6}$-1 X $10^{-5}$ | 7 |
| XXIc | $\underline{R}_C\underline{S}_P$ | | | 2.97 (0.64) | 66.9 (12.2) | 22.5 | 1 X $10^{-6}$-8 X $10^{-6}$ | 7 |
| XXId | $\underline{R}_C\underline{R}_P$ | | | 298 (9) | 5.02 (0.05) | 0.0174 | 1 X $10^{-3}$-1 X $10^{-2}$ | 7 |
| | | | | Housefly-Head Acetylcholinesterase | | | | |
| XXI | racemic | | | 0.228 (0.032) | 39.2 (2.6) | 172 | 5 X $10^{-7}$-4 X $10^{-6}$ | 6 |
| XXIa | $\underline{S}_C\underline{S}_P$ | | | 0.670 (0.035) | 111 (4) | 165 | 5 X $10^{-7}$-5 X $10^{-6}$ | 9 |
| XXIb | $\underline{S}_C\underline{R}_P$ | | | 86.1 (9.5) | 6.08 (0.17) | 0.0706 | 5 X $10^{-4}$-8 X $10^{-3}$ | 7 |
| XXIc | $\underline{R}_C\underline{S}_P$ | | | 0.548 (0.083) | 80.3 (7.9) | 146 | 5 X $10^{-7}$-4 X $10^{-6}$ | 8 |
| XXId | $\underline{R}_C\underline{R}_P$ | | | 195 (20) | 3.13 (0.09) | 0.0160 | 1 X $10^{-3}$-1 X $10^{-2}$ | 7 |

Table 9 cont'd

| No. | Config. | Form | % of Total enzyme | $K_a$ | $k_p$ | $k_i$ | I | No. of points |
|---|---|---|---|---|---|---|---|---|
| | | | | Horse Serum Cholinesterase | | | | |
| XXI | racemic | A | 61 | 25.9 (2.2) | 212 (15) | 8.18 | $5 \times 10^{-6}$-$5 \times 10^{-5}$ | 7 |
| | racemic | B | 39 | 0.670 (0.137) | 2.14 (0.11) | 3.19 | $5 \times 10^{-6}$-$5 \times 10^{-5}$ | 7 |
| XXIa | $\underline{S}_C\underline{S}_P$ | A | 43 | 1.9 | 220 | 120 | $10^{-6}$-$10^{-5}$ | 4 |
| | $\underline{S}_C\underline{S}_P$ | B | 31 | 3.55 (0.49) | 103 (12) | 28.9 | $10^{-6}$-$10^{-5}$ | 6 |
| | $\underline{S}_C\underline{S}_P$ | C | 26 | 0.146 (0.012) | 2.10 (0.18) | 14.4 | $10^{-6}$-$10^{-5}$ | 7 |
| XXIb | $\underline{S}_C\underline{R}_P$ | A | 20 | 3.44 (1.05) | 42.9 (4.9) | 12.5 | $10^{-5}$-$10^{-4}$ | 7 |
| | $\underline{S}_C\underline{R}_P$ | B | 39 | 6.90 (1.31) | 13.5 (1.2) | 1.96 | $10^{-5}$-$10^{-4}$ | 7 |
| | $\underline{S}_C\underline{R}_P$ | C | 41 | 2.25 (0.32) | 1.61 (0.07) | 0.714 | $10^{-5}$-$10^{-4}$ | 7 |
| XXIc | $\underline{R}_C\underline{S}_P$ | | | 1.08 (0.14) | 9.33 (0.87) | 8.63 | $5 \times 10^{-7}$-$5 \times 10^{-6}$ | 6 |
| XXId | $\underline{R}_C\underline{R}_P$ | | | 17.8 (2.1) | 3.81 (0.10) | 0.215 | $10^{-3}$-$10^{-2}$ | 7 |

on its toxicological properties. Of the two asymmetric centers in this molecule, the effect of chirality on anticholinesterase activity and toxicity was much more pronounced at the phosphorus atom than at the 2-butyl carbon atom. On an overall basis, toxicological activity, i.e., toxicity to insects and mice, and plant systemic activity of the individual isomers of XVII appeared to be related to their ability to inhibit stereoselectively the target enzyme, acetylcholinesterase, suggesting that other factors involved in intoxication and detoxication of the individual isomers are similar. As plant systemic insecticides, however, the more toxic (S)-phosphorus isomers were far more effective as translocated insect toxicants than predictable from the activity of the racemic mixture alone. This enhancement of systemic activity, produced by resolution of the isomers, may have practical implications and deserves further study.

The kinetic data presented for compounds XXIa-d must be rationalized on the basis of steric grounds since chemical reactivity arguments are not applicable to rate differences observed between chiral isomers. With the acetylcholinesterases (BAChE and HFAChE), the stereochemical qualities of the $\underline{S}_p$ isomers which are responsible for high affinity ($K_a$) apparently are also responsible for rapid phosphonylation ($k_p$). The converse appears to hold true for the $\underline{R}_p$ isomers since, in general, these gave unfavorable affinity and correspondingly low phosphonylation constants.

On an overall basis, the results indicate that the stereochemical requirements for complex formation and for subsequent phosphonylation are significantly more rigid with AChE than with pseudo ChE. Further, for the two AChE's, stereochemical effects are more noticeable in the inhibition of HFAChE than with BAChE. In spite of these specific differences in the behavior of the three cholinesterases to inhibition by chiral isomers, in general, the order in which the three enzymes are inhibited by the four isomeric inhibitors are similar. This suggests that the stereochemistry associated with the active site of all three enzymes, while different in detail; is, on the whole, similar.

The effect of small, but systematic variation in the size and shape of organophosphorus esters on the inhibition of AChE often has been attributed to the effect of structure modification on the affinity of the inhibitor for the enzyme. On the other hand, the effect of change in the reactivity of the ester on inhibition has been associated with the phosphorylation step. From this study, it appears that both affinity ($K_a$) and phosphonylation ($k_p$) are significantly affected by the stereochemical properties of the chiral phosphorus ester.

## 4. Absolute Configuration of Chiral O-2-Butyl Ethylphosphonothioic Acid.

*Introduction* - In the preceding section, the synthesis, resolution, anticholinesterase activity and toxicological properties of the chiral isomers of *O*-2-butyl *S*-2-(ethylthio)ethyl and *O*-2-butyl *S*-2-(dimethylammonium)ethyl ethylphosphonothioate are discussed. Each of the chiral isomers of these organophosphorus esters was prepared directly from the four corresponding isomers of *O*-2-butyl ethylphosphonothioic acid, resolved *via* the α-phenylethylammonium salt. In light of the profound differences in anticholinesterase and insecticidal activity observed between chiral isomers of these esters, it was of fundamental importance to establish the absolute configuration of the asymmetric centers in the esters. Since both XXIII and XXI were prepared by chemical reactions which did not affect the chiral centers, i.e., the 2-butyl carbon or phosphorus atoms, it was possible to relate absolute configuration of the isomers of these compounds with those of the intermediate acid (XVI). This report is concerned with establishment of the absolute configuration of (-)-*O*-2-butyl (-)-ethylphosphonothioic acid in the form of its α-phenylethylammonium salt (XVIId) by X-ray crystallographic analysis.

*X-Ray Analysis* - (-)-*O*-2-Butyl (-)-ethylphosphonothioic acid (XVIId) bp 80-1° (0.03 mm), $n_D^{25}$ 1.4784, $\alpha_D^{26}$ -33.967° (neat), $[\alpha]_D^{26}$ -9.128° (ethanol) was treated with (-)-α-phenylethylamine to afford the corresponding ammonium salt, XVIId. Appropriate crystals of XVIId were obtained as colorless needles, mp 149-149.5°, $[\alpha]_D^{25}$ -13.718° (ethanol) by slow evaporation of a pentyl acetate solution. *Anal*. Calcd for $C_{14}H_{26}NO_2PS$: C, 55.42; H, 8.64. Found: C, 55.25; H, 8.48. Weissenburg and precession photographs showed monoclinic symmetry and space group $P2_1$. The unit cell constants, a = 11.553(12), b = 6.700(6), c = 11.723(12) A, and β = 107.03(2)°, were determined from a least-square fit of 12 reflections measured on a Picker automatic diffractometer (MoKα, λ = 0.71069 A). The density of the crystal measured by flotation was 1.15 g cm$^{-3}$, which agrees with the value of 1.161 g cm$^{-3}$ calculated for two molecules of $C_{14}H_{26}NO_2PS$ in a unit cell.

Intensity data were calculated on the above diffractor, using MoK$_\alpha$ radiation. Reflections having 2θ values up to 36° (638 unique reflections) were collected by the 2θ-θ scan technique at a scan rate of 1° per minute and a scan range of 1.9°. Background counts of 10 sec were made at each end of the scan.

The structure was solved by the heavy-atom method and refined full matrix least-squares calculations, using 569 reflections which were greater than 1.5σ. The phosphorus and sulfur atoms were treated anisotropically. The refinement converged to a final residue R of

9.6%. All hydrogen atoms were observed on the difference electron-density map, but not included in the refinement. Final atomic coordinates are given in Table 10.

A structural view of XVIId is shown in Figure 8. The quaternary ammonium protons are hydrogen bonded to three neighboring ions, one on the sulfur and two on the oxygen atoms of two different organophosphorus molecules. One of the N-H·····O bonds is shown in Figure 8. The absolute configuration of (-)-*O*-2-butyl (-)-ethylphosphonothioate moiety was determined as $\underline{R}_C\underline{S}_P$, i.e., *R* for carbon and *S* for phosphorus, by relating to the known configurations of the *S*-(-)-α-phenylethylammonium ion and *R*-(-)-2-butyl alcohol.

Based on the assignment $\underline{R}_C\underline{S}_P$ for XVIId, the absolute configurations of the 2-butyl carbon atom and phosphorus atom in (-)-2-butyl *S*-2-(ethylthio)ethyl (-)-ethylphosphonothioate (XVIIId) and (-)-*O*-2-butyl *S*-2-(dimethylammonium)ethyl (-)-ethylphosphonothioate (XXIc) are also assigned the configurations $\underline{R}_C\underline{S}_P$. The configuration of the remaining chiral isomers may be deduced from their respective optical rotations.

*Conclusion* - The absolute configuration of the C and P atoms in the (-)-*O*-2-butyl (-)-ethylphosphonothioate moiety was established by X-ray diffraction analysis as $\underline{R}_C\underline{S}_P$. These results were used to assign the absolute configurations of the C and P atoms in the different chiral isomers of the esters XVIII and XXI.

Table 10. FINAL POSITIONAL PARAMETERS[a] IN S-(-)-α-PHENYLETHYLAMMONIUM SALT OF R-(-)-O-2-BUTYL S-(-)-ETHYLPHOSPHONOTHIOATE

| Atom | x | y | z |
|---|---|---|---|
| S | -0.0053( 7) | 1.0000( 0) | 0.2046( 6) |
| P | -0.0377( 7) | 0.7145(15) | 0.2328( 6) |
| O1 | -0.0119(12) | 0.6464(24) | 0.3627(13) |
| O2 | -0.1762(14) | 0.6592(28) | 0.1758(14) |
| N | -0.0861(15) | 0.2702(27) | 0.4025(14) |
| C1 | -0.2956(24) | 0.3879(46) | 0.3961(23) |
| C2 | -0.2583(19) | 0.4577(42) | 0.5092(20) |
| C3 | -0.3315(24) | 0.6013(44) | 0.5534(23) |
| C4 | -0.4418(23) | 0.6542(49) | 0.4694(25) |
| C5 | -0.4777(24) | 0.5858(47) | 0.3551(25) |
| C6 | -0.3991(23) | 0.4527(43) | 0.3181(21) |
| C7 | -0.2209(20) | 0.2308(46) | 0.3519(19) |
| C8 | -0.2501(20) | 0.0208(46) | 0.3938(19) |
| C9 | -0.3424(24) | 0.8630(60) | 0.0557(25) |
| C10 | -0.2401(22) | 0.7090(52) | 0.0471(23) |
| C11 | -0.2955(28) | 0.4787(63) | 0.0003(30) |
| C12 | -0.3516(38) | 0.5146(59) | -0.1156(41) |
| C13 | 0.0394(23) | 0.5439(46) | 0.1524(21) |
| C14 | 0.1765(22) | 0.5628(44) | 0.2092(21) |
| HC7 | -0.243 | 0.238 | 0.263 |
| HC10 | -0.195 | 0.769 | -0.005 |

[a] Standard deviations given in parentheses are in least significant digits

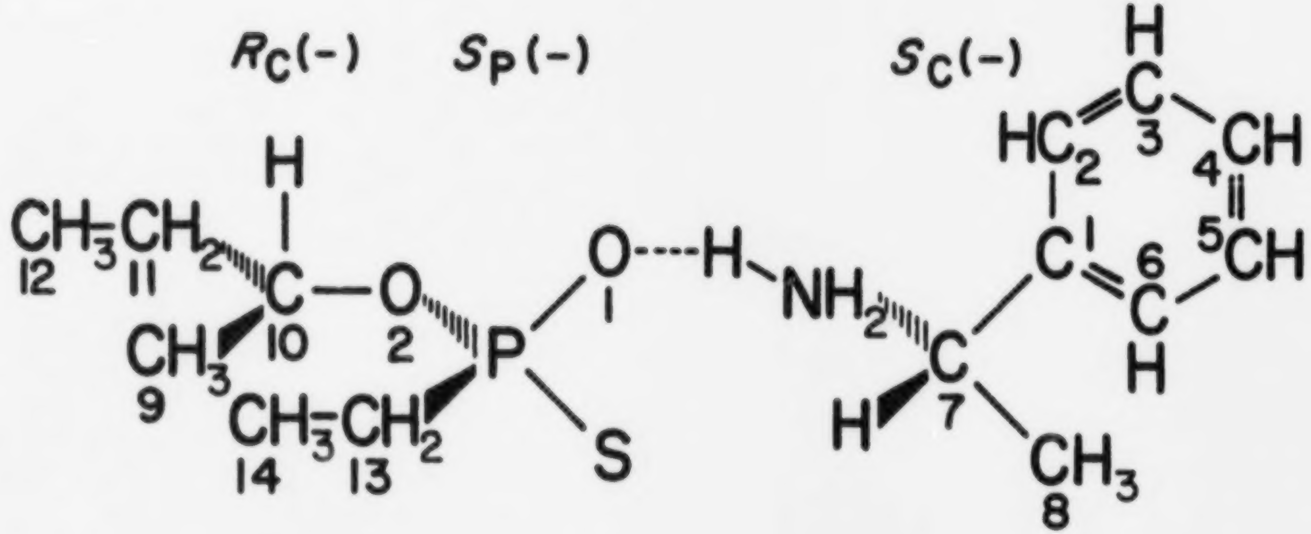


Figure 8. ABSOLUTE CONFIGURATION OF S-(-)-α-PHENYLETHYLAMMONIUM (R)-(-)-O-2-BUTYL (S)-(-)-ETHYLPHOSPHONOTHIOATE (XVIId). THE NUMBERING OF THE ATOMS CORRESPONDS TO THE NUMBERING OF THE ATOMIC COORDINATES GIVEN IN TABLE 10

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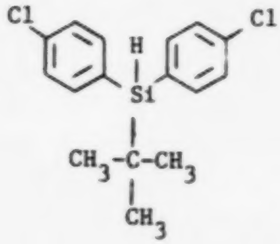
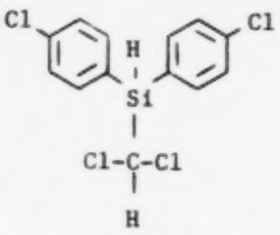
## E. STRUCTURE - ACTIVITY RELATIONSHIPS IN INSECTICIDES

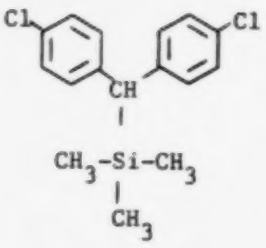
Work on the relationship between chemical structure and biological activity was conducted in a variety of areas. This part of the report summarizes our accomplishments in structure-activity studies of DDT analogs, oxime carbamates and phosphates, S-aryl phosphoramidothioates and *O*,*S*-dialkyl phosphoramidothioates.

### 1. Structure - Activity Correlations in DDT analogs

Introduction - In connection with our studies on the design of selectively toxic and biodegradable insecticides, a number of silicon-containing analogs of DDT were synthesized and evaluated as insecticides. The structure of some of these compounds in which silicon occupies a key position in the molecule is given below.





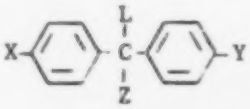


The rationale behind the examination of silicon DDT analogs as biodegradable insecticides resides in the greater instability of the C-Si bond compared to the C-C bond to ionic and radical reactions.

To our disappointment, virtually all of the approximately 20 silicon analogs, including those above, were ineffective against houseflies, showing little or no toxicity at 500 μg/g, alone or in combination with the synergist piperonyl butoxide. The inactivity of these silicon DDT analogs was somewhat surprising, particularly since many of the compounds were direct isosteres of active compounds.

A possible explanation for the ineffectiveness of the silicon analogs may be that these compounds are inherently inactive owing to poor fit at the site of action. Silicon is approximately 50% larger in size than carbon and the Si-C bond (1.86 Å) is considerably longer than the C-C bond (1.48 Å). The effect of the increase in bond length may be seen by comparing the average van der Waal's radii of 2.79 Å and 3.3 Å for the t-butyl and trimethylsilyl moieties, respectively. Therefore, the substitution of a silicon atom in a central position in the DDT analog would cause an increase in the overall size of the molecule in all tetrahedral directions and it is possible that this increase in size prevents interaction of the molecule with the DDT receptor site. Although this may be a plausible explanation for the inactivity of silicon DDT analogs, actually there is little theoretical basis for its support. For this reason, it was decided that an analysis of the relationship between structure of DDT analogs and insecticidal activity was needed for purposes of correlation and prediction of activity.

Theory - The working model that we have adopted for the mode of action of DDT and related compounds is essentially a modification of the model proposed by Holan.[1] The basic premise is that DDT and its analogs fit in a receptor site of a macromolecule, possibly a protein or lipoprotein in a nerve membrane. The receptor site may be visualized as a cavity or a pouch with a limited amount of flexibility and is by no means a rigid structure. Maximum effect occurs when there is maximum interaction between the DDT molecule and the receptor site, particularly with respect to the four key substituents X, Y, L, and Z (structure below) through van der Waal's forces.



For maximum interaction, the overall size of the DDT molecule, i.e., summation of the size of X, Y, L, and Z, is critical and any deviation from this size results in reduced interaction, hence reduced activity. The concept of maximum overall size is illustrated in Figure 1.

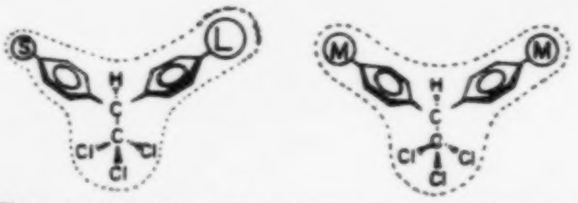


Figure 1. Hypothetical models showing the fit of DDT analogs containing different size ring substituents with the DDT receptor site

M is a substituent approximately the size of Cl, S is smaller than the M and L is larger than M. The point to be made is that good fit with the receptor site is not restricted to symmetrical molecules but also may be obtained with unsymmetrical analogs, as long as the overall size of the molecule remains within the flexible framework of the receptor site. This concept differs from the model of Holan[1] where the size of each substituent is assumed to be independent of each other and greater reliance is placed on shape symmetry.

Basically, our model informs us that as the summation of the size of X, Y, L, and Z increases, interaction with the receptor increases, eventually reaching a maximum from which point increase in substituent size results in decreased interaction. Assuming that interaction or fit with the hypothetical receptor site is directly related to insect toxicity, it is possible now to correlate insecticidal activity with free energy parameters on a quantitative basis.

Although several different parameters may be used to estimate the size of a substituent, e.g., van der Waal's radii, etc., we chose to use Taft's steric substituent parameter[2] $E_s$ owing to the belief that fit at the receptor site should be governed promarily by steric effects. Keeping in mind that receptor interaction should approach a maximum and then decrease with increase in the steric parameter $E_s$, the most reasonable approximation between toxicity and $E_s$ should be parabolic in nature. Therefore, the mathematical model to express toxicity in terms of $E_s$ with change in a single substituent X may be expressed by the following equation where $\alpha$, $\beta$,

$$\text{Log } LD_{50} = \alpha + \beta \, E_s^X + \gamma \, [E_s^X]^2 \qquad (1)$$

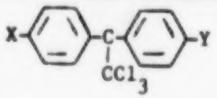
and $\gamma$ are constants. A generalized equation for changes in more than one substituent would be represented by equation 2 where $\alpha'$, $\beta'$, and $\gamma'$ are a new set of constants and i denotes substituent

$$\text{Log } LD_{50} = \alpha' + \beta' \, \overset{i}{\Sigma} E_s + \gamma' \, \overset{i}{\Sigma} [E_s]^2 \qquad (2)$$

X, Y, L, and Z.

The data presented in Tables 1 and 2 were subjected to multiple regression analysis. In accordance with our model, the variables $\Sigma E_s$ and $\Sigma E_s^2$ were forced into the regression equation and other variables (cf Tables 1 and 2) which might contribute significantly to the regression sums of squares at the 0.05 level of probability were entered into the analysis. The data also were analyzed by a program which allowed the stepwise addition of significant variables but in no case did any variable or appropriate combination contribute more significantly to the regression equation than $\Sigma E_s + \Sigma E_s^2$, as anticipated by the model. Besides $E_s$ the free energy parameters used in the analysis were Hansch's $\pi$ constant for hydrophobic bonding,[3] Taft's polar substituent constant[2] $\sigma^*$, and the resonance (R) and field constants (F) of Swain and Lupton[4].

Table 1. EFFECT OF VARIATIONS IN THE X AND Y POSITIONS ON THE INSECTICIDAL ACTIVITY OF DDT ANALOGS

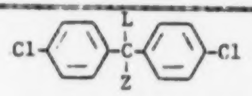


| Compound | X | Y | $E_s^X$ | $E_s^Y$ | $\pi^X$ | $\pi^Y$ | $R^X$ | $R^Y$ | $F^X$ | $F^Y$ | $LD_{50}$ (μg/g) Housefly (alone) | $LD_{50}$ (μg/g) Housefly (+ P.B.) | $LC_{50}$ (ppm) *Culex fatigans* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | H | H | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2900 | 575 | 1.1 |
| 2 | F | F | -0.46 | -0.46 | 0.15 | 0.15 | -0.34 | -0.34 | 0.71 | 0.71 | 30.5 | 17.0 | 0.074 |
| 3 | Cl | Cl | -1.16 | -1.16 | 0.70 | 0.70 | -0.16 | -0.16 | 0.69 | 0.69 | 14 | 5.5 | 0.07 |
| 4 | Br | Br | -1.36 | -1.36 | 1.02 | 1.02 | -0.18 | -0.18 | 0.73 | 0.73 | 27 | 10.5 | 0.074 |
| 5 | I | I | -1.72 | -1.72 | 1.27 | 1.27 | -0.20 | -0.20 | 0.67 | 0.67 | 35 | 14 | 1.4 |
| 6 | $CH_3$ | $CH_3$ | -1.24 | -1.24 | 0.52 | 0.52 | -0.14 | -0.14 | -0.05 | -0.05 | 100 | 17.5 | 0.081 |
| 7 | $C_2H_5$ | $C_2H_5$ | -1.62 | -1.62 | 1.00 | 1.00 | -0.11 | -0.11 | -0.06 | -0.06 | 70 | 29.0 | 0.18 |
| 8 | $OCH_3$ | $OCH_3$ | -1.08 | -1.08 | -0.04 | -0.04 | -0.50 | -0.50 | 0.41 | 0.41 | 45 | 3.5 | 0.067 |
| 9 | $OC_2H_5$ | $OC_2H_5$ | -1.17 | -1.17 | 0.50 | 0.50 | -0.44 | -0.44 | 0.36 | 0.36 | 7.0 | 1.75 | 0.04 |
| 10 | $CH_3$ | H | -1.24 | 0 | 0.52 | 0 | -0.14 | 0 | -0.05 | 0 | >500 | 33.0 | 0.51 |
| 11 | Cl | $CH_3$ | -1.16 | -1.24 | 0.70 | 0.52 | -0.16 | -0.14 | 0.69 | -0.05 | 62.5 | 6.5 | 0.031 |
| 12 | F | $CH_3$ | -0.46 | -1.24 | 0.15 | 0.52 | -0.34 | -0.14 | 0.71 | -0.05 | 250 | 25 | 0.059 |
| 13 | Br | $CH_3$ | -1.36 | -1.24 | 1.02 | 0.52 | -0.18 | -0.14 | 0.73 | -0.05 | 32.5 | 1.75 | 0.04 |
| 14 | I | $CH_3$ | -1.72 | -1.24 | 1.27 | 0.52 | -0.20 | -0.14 | 0.67 | -0.05 | 120 | 22.2 | 0.044 |
| 15 | F | $OCH_3$ | -0.46 | -1.08 | 0.15 | -0.04 | -0.34 | -0.50 | 0.71 | 0.41 | 47 | 11.5 | 0.47 |

Table 1 continued

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16 | Cl | $OCH_3$ | -1.16 | -1.08 | 0.70 | -0.04 | -0.16 | -0.50 | 0.69 | 0.41 | 41.5 | 7.0 | 0.058 |
| 17 | $CH_3$ | $OCH_3$ | -1.24 | -1.08 | 0.52 | -0.04 | -0.14 | -0.50 | -0.05 | 0.41 | 23.5 | 4.9 | 0.085 |
| 18 | $CH_3$ | $OC_2H_5$ | -1.24 | -1.17 | 0.52 | 0.50 | -0.14 | -0.44 | -0.05 | 0.36 | 9 | 1.7 | 0.13 |
| 19 | $OCH_3$ | $OC_2H_5$ | -1.08 | -1.17 | -0.04 | 0.50 | -0.50 | -0.44 | 0.41 | 0.36 | 16 | 3.7 | 0.039 |
| 20 | $SCH_3$ | $SCH_3$ | -1.29 | -1.29 | 0.62 | 0.62 | -0.19 | -0.19 | 0.33 | 0.33 | 225 | 17.0 | 0.25 |
| 21 | $SCH_3$ | $OCH_3$ | -1.29 | -1.08 | 0.62 | -0.04 | -0.19 | -0.50 | 0.33 | 0.41 | 32 | 4.0 | 0.11 |
| 22 | $SCH_3$ | $OC_2H_5$ | -1.29 | -1.17 | 0.62 | 0.50 | -0.19 | -0.44 | 0.33 | 0.36 | 32 | 2.8 | 0.7 |
| 23 | $CH_3$ | $C_2H_5$ | -1.24 | -1.62 | 0.52 | 1.00 | -0.14 | -0.11 | -0.05 | -0.06 | 11 | 3.0 | 0.08 |
| 24 | $OCH_3$ | $CH(CH_3)_2$ | -1.08 | -2.32 | -0.04 | 1.30 | -0.50 | -0.14 | 0.41 | -0.10 | 160 | 61.5 | 0.32 |
| 25 | Cl | $CH(CH_3)_2$ | -1.16 | -2.32 | 0.70 | 1.30 | -0.16 | -0.14 | 0.69 | -0.10 | 215 | 93.5 | 0.15 |

Table 2. EFFECT OF VARIATION IN THE L AND Z POSITIONS ON THE INSECTICIDAL ACTIVITY OF DDT ANALOGS



| Compound | L | Z | $E_s^L$ | $E_s^Z$ | $\pi^L$ | $\pi^Z$ | $\sigma^{*L}$ | $\sigma^{*Z}$ | $LD_{50}$ (µg/g) Houseflies | $LC_{50}$ (ppm) Culex fatigans |
|---|---|---|---|---|---|---|---|---|---|---|
| 3 | H | $CCl_3$ | 0 | -3.30 | 0 | 1.70 | 0 | 1.00 | 2.0 | 0.070 |
| 26 | H | $CBr_3$ | 0 | -3.67 | 0 | 2.32 | 0 | 1.00 | >500 | 0.550 |
| 27 | H | $CF_3$ | 0 | -2.40 | 0 | 0 | 0 | 0.92 | >500 | 0.250 |
| 28 | H | $CHCl_2$ | 0 | -2.78 | 0 | 1.32 | 0 | 0.70 | 20 | 0.038 |
| 29 | H | $CHF_2$ | 0 | -1.91 | 0 | 0.18 | 0 | 0.70 | >500 | 0.950 |
| 30 | H | CHClF | 0 | -2.35 | 0 | 0.85 | 0 | 0.70 | 155 | 0.120 |
| 31 | F | $CCl_3$ | -0.46 | -3.30 | -0.17 | 1.70 | 1.10 | 1.00 | 125 | 0.092 |
| 32 | F | $CHCl_2$ | -0.46 | -2.78 | -0.17 | 1.32 | 1.10 | 0.70 | 4.1 | 0.024 |
| 33 | F | CHClBr | -0.46 | -2.94 | -0.17 | 1.52 | 1.10 | 0.70 | 2.8 | 0.021 |
| 34 | F | CHClF | -0.46 | -2.34 | -0.17 | 0.85 | 1.10 | 0.70 | 220 | 0.110 |
| 35 | F | $CHBr_2$ | -0.46 | -3.00 | -0.17 | 1.72 | 1.10 | 0.70 | 7.0 | 0.022 |
| 36 | F | CHBrF | -0.46 | -2.51 | -0.17 | 0.93 | 1.10 | 0.70 | 70 | 0.085 |
| 37 | F | $CHBrCH_3$ | -0.46 | -2.41 | -0.17 | 1.64 | 1.10 | 0.30 | 9.0 | 0.070 |
| 38 | F | $CHF_2$ | -0.46 | -1.91 | -0.17 | 0.18 | 1.10 | 0.70 | >500 | 0.810 |

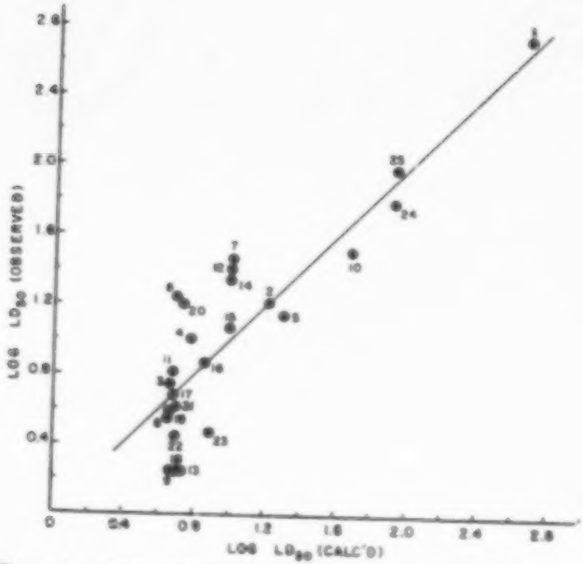


Figure 2. Correlation between observed synergized housefly toxicity and toxicity calculated according to eq 4 for variation in X and Y positions of DDT analogs.

A composite of data developed in two different laboratories over a period of about 15 years[5,6] was used in the analysis of mosquito toxicity. For the 25 compounds in Table 1, stepwise selection of variables by significance did not provide any significant correlation between toxicity and any of the parameters. Forcing $\Sigma E_s$ and $\Sigma E_s^2$ in the regression analysis resulted in equation 5.

$$\text{Log } LC_{50} = 0.99\ (\pm\ 0.29)\ \Sigma E_s + 0.46\ (\pm\ 0.14)\ \Sigma E_s^2 \qquad (5)$$

| $\underline{n}$ | $\underline{r}$ | $\underline{s}$ |
|---|---|---|
| 25 | 0.595 | 0.4 |

However, when the analysis was restricted to the compounds evaluated in the early study[5], i.e. compounds **1-11** and the compound where X is Cl and Y is H, a satisfactory correlation was obtained (equation 6). The relationship between observed $LC_{50}$ and $LC_{50}$ calculated according to equation 6 is shown graphically in Figure 3.

$$\text{Log } LC_{50} = 1.63\ (\pm\ 0.21)\ \Sigma E_s + 0.93\ (\pm\ 0.12)\ \Sigma E_s^2 \qquad (6)$$

| $\underline{n}$ | $\underline{r}$ | $\underline{s}$ |
|---|---|---|
| 12 | 0.933 | 0.21 |

Effect of Variation in X and Y Positions For the series of 25 symmetrical and unsymmetrical DDT analogs in which X and Y are varied (Table 1), the following equations relating toxicity ($LD_{50}$) against houseflies, with and without the synergist piperonyl butoxide, with $E_s$ were formulated. Without synergist:

$$\text{Log } LD_{50} = 3.24\ (\pm\ 0.35) + 1.52\ (\pm\ 0.22)\ \Sigma E_s + 0.65\ (\pm\ 0.14)\ \Sigma E_s^2 \tag{3}$$

| n | r | s |
|---|---|---|
| 24 | 0.736 | 0.41 |

With synergist:

$$\text{Log } LD_{50} = 2.69\ (\pm\ 0.25) + 1.85\ (\pm\ 0.22)\ \Sigma E_s + 0.85\ (\pm\ 0.11)\ \Sigma E_s^2 \tag{4}$$

| n | r | s |
|---|---|---|
| 25 | 0.874 | 0.31 |

n is the number of compounds in the analysis, r is the correlation coefficient and s is the standard error.

Comparison of equations 3 and 4 shows that the use of piperonyl butoxide significantly improves the correlation between housefly toxicity and the steric substituent constant $E_s$. Since piperonyl butoxide is regarded as an inhibitor of microsomal mixed function oxidase, the improved correlation between synergized toxicity and $E_s$ serves to point out the importance of the effect of oxidative metabolism on the insecticidal activity of DDT analogs. The relationship between synergized housefly toxicity and $E_s$ is presented graphically (Figure 2) by plotting log $LD_{50}$ observed against log $LD_{50}$ calculated according to equation 4. Several of the compounds notably 6, 7, 12, 14, and 20, appear to be considerably less toxic than expected from the equation, while a few (9, 13, and 18) are more toxic. Since enzymatic dehydrochlorination of DDT and related compounds is not blocked by piperonyl butoxide, it is possible that these compounds deviate from the calculated fit because of differences in their susceptibility to dehydrochlorination by DDT dehydrochlorinase. However, it should be pointed out that the deviation of these points from the line is not as serious as may appear from casual observation of the figure since most of the deviations are in the region where high toxicity is expected. For example, the largest deviation occurs with compounds with a calculated $LD_{50}$ in the range of 4-6.5 μg/g (log $LD_{50}$ 0.3-1.2). Considering the inherent variability usually found in toxicity data and the number of compounds in the analysis, the fit of the points to the regression line is quite good.

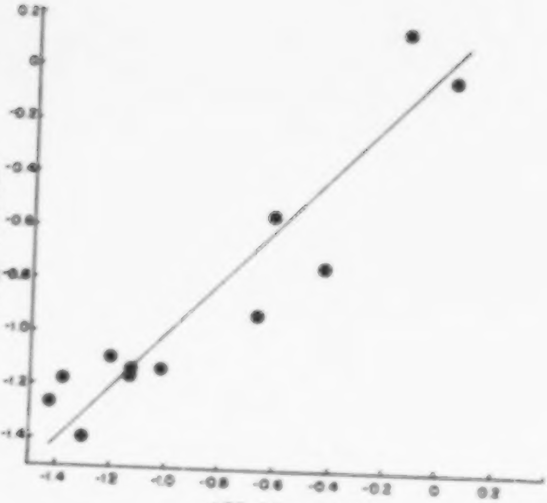


**Figure 3.** Correlation between observed mosquito larvae toxicity and toxicity calculated according to eq 6 for variation in X and Y positions in DDT analogs.

On the other hand, analysis of the mosquito toxicity data provided in the more recent study of DDT analogs,[6] i.e., compounds 12-25, gave poor correlations between toxicity and $E_s$ ($r$ = 0.433). The basis for the poor correlation, in light of the highly satisfactory correlation obtained for the other compounds, is not known. It is noteworthy that 1-9 are symmetrical compounds while the remaining, except for 20, are unsymmetrical DDT analogs. Whether asymmetry in the molecule is responsible for the poor correlation is conjectural at this time. It should be pointed out, however, that asymmetry did not affect the correlation on houseflies.

In general, correlation of toxicity with the steric substituent constant $E_s$ was reasonably satisfactory with variation in the X and Y positions when oxidative metabolism was minimized by piperonyl butoxide. The successful use of the sum of the steric effects in the X and Y position in correlating insecticidal activity suggests that the DDT receptor site is quite flexible and is not as rigid as depicted by Holan.[1] This model adequately explains why DDT analogs which contain a substituent smaller than chlorine in the X position and a substituent larger than chlorine in the Y position are toxic. Evidently, the degree of interaction between the substituents X and Y with the receptor site largely depends upon the sum of the steric effects produced by both X and Y as suggested in Figure 1. A graphic illustration of this concept is presented in Figure 4 where synergized log $LD_{50}$ against houseflies is plotted against $E_s$ for different substituents in one position (X) and the rest of the molecule is held constant (Y = methyl). With the exception of the point representing the symmetrical dimethyl analog (X = Y = methyl), the plot is strikingly similar to the well known potential energy diagram for diatomic molecules. The figure shows that there is a region where maximum interaction occurs between the compound and the receptor site and interaction decreases as the sum of $E_s$ for X and Y is above or below this region.

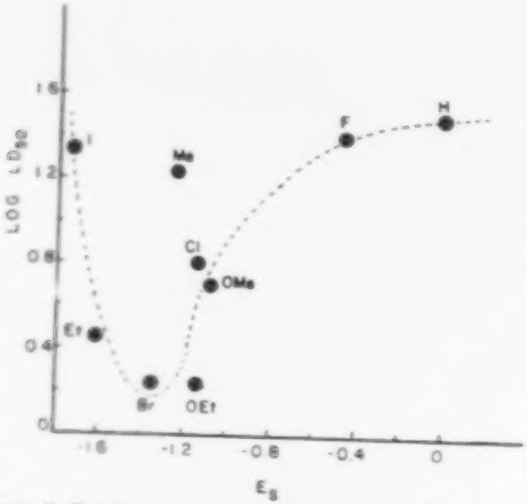


Figure 4. Relationship between observed synergized housefly toxicity and $E_s$ for substituent X for 1,1,1-trichloro-p-methyl-p′-X-diphenylethanes.

Effect of Variation in L and Z Positions - The importance of the size of the downward group Z on the insecticidal activity of DDT analogs is well known. Holan[1] has indicated that the downward projection of DDT and the corresponding dichlorocyclopropane analogs are similar and of optimum size (6.0-6.3 Å). Although downward projection into a membrane cavity to account for the effect of Z on toxicity has been useful in correlating structure with insecticidal activity, this type of projection analysis has ignored the effect of the simultaneous presence of the group in the L position. To illustrate this point, the replacement of the benzylic hydrogen in DDT by fluorine (31) does not effectively change the downward projection of the trichloromethyl moiety; however, housefly toxicity drops by a factor of about 63. On the other hand, replacement of the benzylic hydrogen in DDT (28) with fluorine resulted in a compound (32) which was only two-fold less toxic than DDT. From these examples, it is evident that the effect of substituents on the α-carbon should be examined together and not separately.

Toxicological properties and the various parameters for DDT analogs where L and Z are varied are given in Table 2. Analysis of the data provided equation 7 for unsynergized housefly toxicity.

$$\text{Log } LD_{50} = 14.14\ (\pm 4.0) + 9.36\ (\pm 3.0)\ \Sigma E_s + 1.12\ (\pm 0.47)\ \Sigma E_s^{\,2} + 4.31\ (\pm 1.37)\ \Sigma \sigma^* \qquad (7)$$

| *n* | *r* | *s* |
|---|---|---|
| 9 | 0.856 | 0.31 |

For mosquito larvae, equations 8 and 9 were obtained.

$$\text{Log } LC_{50} = 5.29\ (\pm 1.06) + 4.90\ (\pm 0.92)\ \Sigma E_s + 0.76\ (\pm 0.16)\ \Sigma E_s^{\,2} + 1.76\ (\pm 0.43)\ \Sigma \sigma^* \qquad (8)$$

| *n* | *r* | *s* |
|---|---|---|
| 14 | 0.889 | 0.29 |

$$\text{Log } LC_{50} = 7.27\ (\pm 0.72) + 6.03\ (\pm 0.57)\ \Sigma E_s + 0.76\ (\pm 0.09)\ \Sigma E_s^{\,2} + 2.27\ (\pm 0.26)\ \Sigma \sigma^* + 0.37\ (\pm 0.08)\ \Sigma \pi^2 \qquad (9)$$

| *n* | *r* | *s* |
|---|---|---|
| 14 | 0.970 | 0.16 |

While significant correlation was not obtained when each of the parameters was used alone, the combination of $E_s$ and $\sigma^*$ was highly significant and indicated that steric and polar effects contributed to interaction of L and Z with the receptor site in both houseflies and mosquito larvae. Against mosquito larvae, significant improvement in correlation was obtained by the introduction of a $\pi^2$ term (equation 9). Although the coefficient for this term (0.37) is small, it does point out the importance of the lipophilic character of L and Z on toxicity to mosquito larvae.

For groups on the α-carbon atom, equations 7, 8, and 9 predict that higher log $LD_{50}$ or log $LC_{50}$ (lower toxicity) will be obtained for substituents with approximately equal $E_s$ values but larger $\sigma^*$ values. This is quite surprising since virtually all of the more toxic DDT analogs contain polar groups in the Z (or L) position, i.e., groups with reasonably large positive $\sigma^*$ constants. However, of the compounds examined, $\sigma^*$ for the Z substituent resides in a rather narrow range compared to the corresponding $E_s$ values and, therefore, any conclusions concerning the effect of $\sigma^*$ on toxicity must be tempered with caution. It is possible that there is also a value of $\sigma^*$ where maximum toxicity effects would be observed, similar to that for $E_s$. Unfortunately, however, data was not available for DDT analogs with a diverse range of $\sigma^*$ values for the Z substituent, particularly those with negative values.

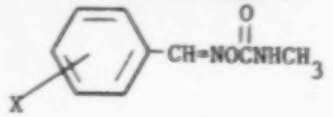
Conclusion - Overall, the steric substituent constant $E_s$ appears to be the single most important parameter for the correlation of housefly and mosquito larvae toxicity when substituents are varied in the X, Y, L, and Z positions in DDT analogs. The successful use of $\Sigma E_s$ and $\Sigma E_s^2$ in correlating insecticidal activity with structure strongly idicates that there is a reasonable amount of flexibility in the DDT receptor site. The receptor site may be visualized as a flexible cavity which can accommodate DDT analogs of varying dimensions as long as the overall size of the molecule does not deviate substantially from that of DDT. Compounds possessing the same geometry as that of DDT but fall outside the range of maximum interaction because of their overall size, i.e., smaller or larger, therefore are not expected to be toxic. The organosilicon analogs of DDT, mentioned earlier, probably fall into this catagory.

There is evidence to indicate that the effect of ring substituents is not independent of that of substituents on the $\alpha$-carbon atom. For example, the toxicity of Perthane [1,1-dichloro-2,2-bis-(p-ethylphenyl(ethane] to houseflies synergized with piperonyl butoxide is approximately twice that of synergized DDT. In comparison, the toxicity of synergized 1,1,1-trichloro-2,2-bis-(p-ethylphenyl) ethane, the p-ethyl analog of DDT, is only 1/6th that of synergized DDT. This suggests that when group X or Y or both are altered, insecticidal activity may be maintained by appropriate alteration of L or Z. The $E_s$ values for the chloro and ethyl moieties are -1.16 and -1.62, respectively. Changing the ring substituent from chloro to ethyl, therefore, can be compensated for by substituting dichloromethyl ($E_s$ = -2.78) for trichloromethyl ($E_s$ = -3.30) in the $\alpha$-position.

It is obvious that a large number of new DDT analogs still remain to be synthesized and examined for insecticidal activity. The model that we have developed in predicting the insecticidal activity of DDT analogs is by no means infallible but it provides a more systematic approach to the design of new compounds.

## 2. Structure, Reactivity, and Biological Activity of O-(Methylcarbamoyl)oximes of Substituted Benzaldehydes

**Introduction** - Interest in oxime carbamates originated from the outstanding insecticidal activity demonstrated by such compounds as 2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamoyl)oxime (aldicarb), *exo*-3-chloro-*endo*-6-cyano-2-norbornanone *O*-(methylcarbamoyl)oxime (Tranid®, Union Carbide), 2-oximino-1,3-dithiolane methylcarbamate (American Cyanamid) and other oxime carbamates and phosphates. Owing to the wide variation in structure of the above oxime carbamates, a systematic examination of these compounds was initiated in an attempt to establish the relationship between structure and reactivity with anticholinesterase and insecticidal activity. This part of the report presents results from a study of a series of O-(methylcarbamoyl)oximes of substituted benzaldehydes of general structure shown below.



**Synthesis** - All substituted benzaldoximes were prepared in the conventional manner from the appropriately substituted benzaldehyde and hydroxylamine. Methylcarbamates were prepared by reaction between the benzaldoxime and methylisocyanate using triethylamine as catalyst.

**Alkaline Hydrolysis** - Table 3 provides data for the pseudo 1st-order hydrolysis constants ($k_b$) in phosphate buffer at pH 11.95, anticholinesterase activity ($I_{50}$), and toxicity to the housefly of the carbamate alone and synergized with 1:5 parts piperonyl butoxide.

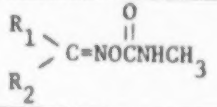
Linear regression analysis of $k_b$ values for the *meta*- and *para*-substituted oxime carbamates produced the equation 10 where r is

$$\log 10\ k_b = 0.908\sigma + 0.271 \qquad (r = 0.98) \qquad (10)$$

the correlation coefficient. Use of the regression equation to calculate new sigma constants gave values of 0.61 and 0.92 for *m*-nitro and *p*-nitro, respectively. These values are in excellent agreement with the sigma values of 0.67 and 0.92 for *m*-nitro and *p*-nitro substituents calculated by linear regression analysis (equation 11) of data obtained by Brady and Goldstein[7] for the ionization constants of substituted benzaldoximes:

$$pK_a = -0.774\,\sigma + 10.66 \qquad (r = 0.99) \qquad (11)$$

Table 3. PHYSICAL AND BIOLOGICAL PROPERTIES OF *O*-(METHYLCARBAMOYL)OXIMES OF SUBSTITUTED BENZALDEHYDES AND RELATED KETONES



$R_1R_2C{=}NOC(O)NHCH_3$

| | $R_1$ | $R_2$ | Oxime isomer | $k_b$ ($min^{-1}$) | $I_{50}$ (*M*) | $LD_{50}$ µg/g Alone | $LD_{50}$ µg/g 1:5 P.B. |
|---|---|---|---|---|---|---|---|
| 39 | phenyl | H | syn | 0.175 | $4.6 \times 10^{-5}$ | >500 | >50 |
| 40 | *p*-methylphenyl | H | syn | 0.131 | - | - | - |
| 41 | *o*-ethylphenyl | H | - | 0.188 | $2.6 \times 10^{-5}$ | >500 | 50 |
| 42 | *o*-isopropylphenyl | H | - | 0.222 | $1.5 \times 10^{-5}$ | >500 | 40 |
| 43 | *m*-isopropylphenyl | H | - | 0.167 | $7.2 \times 10^{-6}$ | >500 | >50 |
| 44 | *p*-isopropylphenyl | H | syn | 0.133 | $3.7 \times 10^{-5}$ | >500 | >100 |
| 45 | *o*-*t*-butylphenyl | H | - | 0.226 | $4.5 \times 10^{-5}$ | >500 | >50 |
| 46 | 2,4,6-trimethylphenyl | H | syn | 0.185 | $5.5 \times 10^{-5}$ | >500 | >50 |
| 47 | *o*-bromophenyl | H | syn | 0.596 | $3.0 \times 10^{-5}$ | >500 | >100 |
| 48 | *p*-bromophenyl | H | syn | 0.332 | $3.8 \times 10^{-5}$ | >1000 | >100 |
| 49 | *o*-nitrophenyl | H | - | - | $7.1 \times 10^{-5}$ | >500 | >100 |
| 50 | *m*-nitrophenyl | H | syn | 0.622 | $8.3 \times 10^{-6}$ | >1000 | 40 |
| 51 | *p*-nitrophenyl | H | syn | 1.190 | $1.4 \times 10^{-5}$ | >1000 | >100 |

Table 3 continued.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **52** | o-methoxyphenyl | H | syn | 0.119 | $3.1 \times 10^{-5}$ | >500 | >50 |
| **53** | o-ethoxyphenyl | H | syn | 0.111 | $3.7 \times 10^{-5}$ | >1000 | >50 |
| **54** | o-propoxyphenyl | H | - | 0.151 | $9.1 \times 10^{-6}$ | >500 | >50 |
| **55** | o-methylthiophenyl | H | syn | 0.325 | $4.9 \times 10^{-6}$ | >500 | >50 |
| **56** | o-$CF_3$-phenyl | H | - | - | - | >500 | >50 |
| **57** | 3,4-methylenedioxyphenyl | H | syn | 0.114 | $8.1 \times 10^{-5}$ | >500 | >50 |
| **58** | 2-$CH_3$S-4,5-methylenedioxyphenyl | H | - | - | $1.7 \times 10^{-5}$ | >500 | >50 |
| **59** | phenyl | thienyl | - | - | $2.8 \times 10^{-5}$ | >500 | 20 |
| **60** | p-fluorophenyl | cyclobutyl | - | - | $1.7 \times 10^{-5}$ | >500 | 40 |
| **61** | p-fluorophenyl | cyclopropyl | - | - | $6.8 \times 10^{-6}$ | >500 | 12.5 |
| **62** | thienyl | cyclopropyl | - | - | $1.5 \times 10^{-6}$ | 75 | 10 |

The values of 0.67 and 0.92 for m-nitro and p-nitro imply that field effects are somewhat less and resonance effects somewhat greater in substituted benzaldoximes than in benzoic acids. Unfortunately, there were not enough meta- and para-substituents examined to subject the $k_b$ values to correlation with the field and resonance constants of Swain and Lupton[4].

The ortho-substituted compounds were not included in the analysis above because of steric or "ortho" effects[2]. However, $k_b$ values for these compounds were well correlated with the $\sigma_o$ constants of Tribble obtained from nmr spectra of ortho-substituted phenols. Linear regression analysis of the data provided the equation 12 below. The value of the reaction constant $\rho$ is 0.758 for the ortho-

$$\log 10\ k_b = 0.758\ \sigma_o + 0.355 \qquad (r = 0.95) \tag{12}$$

substituted oxime carbamates compared to 0.906 for the meta- and para-substituted compounds. For the meta- and para-substituted series all of the compounds except the m-isopropyl compound were in the syn configuration and, therefore, the $\rho$ value is essentially that of the syn isomers. However, in the case of the ortho series the configuration of the ethyl, isopropyl, and t-butyl derivatives was not known with certainty and, therefore, it is possible that $\rho$ in this series was obtained from hydrolysis data involving syn-anti pairs. The difference in $\rho$ constants does not necessarily imply a different mechanism for hydrolysis since the differences in $k_b$ values for ortho- and para-substituents are small enough to be accounted for entirely by steric inhibition of resonance by substituents in the ortho position.

Cholinesterase Inhibition - Anticholinesterase data in Table 3 show that all of the benzaldoxime methylcarbamates were amazingly similar in their ability to inactivate fly-head cholinesterase, i.e. $I_{50}$ values in the neighborhood of $10^{-5}$ M with a range between $9 \times 10^{-5}$ to $5 \times 10^{-6}$ M. This is in direct contrast to the substituted phenyl N-methylcarbamates which have been found to vary as much as $10^5$ in their $I_{50}$ values[9], depending on the nature and position of the substituent. Closer examination of the data reveals an approximate but direct relationship between anticholinesterase activity and alkaline hydrolysis rates for the meta- and para-substituted compounds. Aside from the m-isopropyl derivative which is a much stronger inhibitor than predicted by its hydrolysis rate, a fair correlation between log $k_b$ and -log $I_{50}$ is obtained. The direct relationship between anticholinesterase activity and reactivity as measured by alkaline hydrolysis rates is analogous to results obtained in a previous study of O-(methylcarbamoyl)oximes of ring-substituted acetophenones[10].

The narrow range of anticholinesterase activity cited above and the direct correlation between anticholinesterase activity and $k_b$ of the benzaldoxime methylcarbamates suggest that steric properties of the molecule and interaction of the carbamate and enzyme to form the complex are of lesser importance in the inhibition process than the carbamylation reaction. Thus, the situation appears to be similar to cholinesterase inhibition by organophosphorus esters but unlike substituted phenyl methylcarbamates where it has been demonstrated that variation in inhibition is more a function of degree of complex formation rather than carbamylation[11].

No relationship between anticholinesterase activity and hydrolysis rates or any other parameter was evident with the *ortho*-substituted *O*-(methylcarbamoyl)-benzaldoximes. The order of inhibition for the ortho alkyl substituents is isopropyl > ethyl > *t*-butyl ≈ methyl, where methyl is for the 2,4,6-trimethyl compound (46). Although the range in $I_{50}$ values between the isopropyl (42) and 2,4,6-trimethyl compounds is small ($I_{50}$ $1.5 \times 10^{-5}$ to $5.5 \times 10^{-5}$), the lower activity of the *t*-butyl (45) derivative may be due to steric effects, possibly to inhibition of rotation. The order of inhibition with the *ortho*-alkoxy analogs was propoxy > ethoxy ≈ methoxy but again difference in activity was slight.

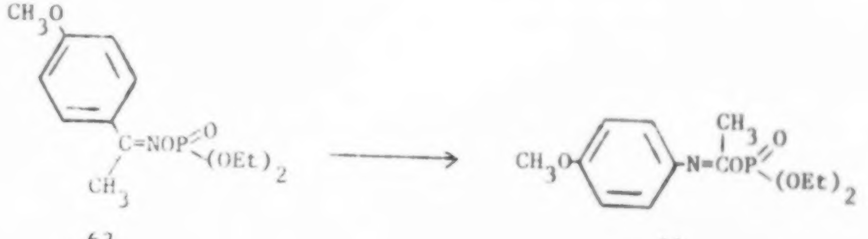
*Toxicity* - None of the ring substituted *O*-(methylcarbamoyl)benzaldoximes were toxic to house flies when used alone. Cotreatment with 5 parts of piperonyl butoxide to 1 part of carbamate increased toxicity to house flies in some cases but unusual examples of synergism were not achieved. The *m*-nitro- (50) and the *o*-isopropylbenzaldoxime (42) carbamates were the only compounds which showed significant activity against house flies with piperonyl butoxide. Overall, the poor insecticidal activity of these compounds was disappointing but not entirely unexpected owing to the generally weak activity previously found for the *O*-(methylcarbamoyl)oximes of substituted acetophenones.

Four *O*-(methylcarbamoyl)ketoximes also are listed in Table 3 as a continuation of previous work with these compounds[10]. The cyclopropyl thienyl ketoxime carbamate was the only compound in the entire series which proved to be significantly toxic without piperonyl butoxide. All of the ketoxime carbamates, however, were significantly synergized by means of piperonyl butoxide and these compounds were included in this paper to point out the variety of organic groups which may be incorporated into an oxime carbamate.

*Conclusion* - This series of substituted *O*-(methylcarbamoyl)benzaldoximes is less toxic to insects and slightly less active as inhibitors of cholinesterase than the phenyl *N*-methylcarbamates. However, they are more stable to alkaline hydrolysis. The structure-activity study indicates that electron-withdrawing substituents increase anticholinesterase activity which is opposite to the relationship found with the phenyl *N*-methylcarbamates. This would be logical if there existed an optimum reactivity for the reaction with cholinesterase; the multi-step reaction sequence for the inhibition process lends itself to such a theory.

### 3. Substituted Acetophenone *O*-(diethylphosphoryl)Oximes. Thermal Rearrangement and Biological Activity.

Introduction - In an early study concerned with the relation between structure of a series of *O*-(diethyl phosphoryl)oximes of ring-substituted acetophenones and biological activity, preliminary evidence was presented which indicated that oxime phosphate esters containing electron-donating substituents on the benzene ring were unstable and rearranged to the imidoyl phosphate as exemplified below with the *p*-methoxy derivative (**63**).



**63** **64**

Further, since both rates of alkaline hydrolysis and anticholinesterase activity of the various oxime phosphate esters were linearly correlated with the electron-donating tendency of the substituent, the suggestion was made that it was the imidoyl phosphate (**64**) formed from the rearrangement of the oxime phosphate (**63**) that was involved in hydrolysis and cholinesterase inhibition. It was not clear, however, whether **64** was formed during the hydrolysis and inhibition process or whether it was formed during the preparation of **63**, prior to measurement of hydrolysis or anticholinesterase rates. Owing to the possible implication of the imidoyl phosphate in the mode of action of oxime phosphate esters, further study of the rearrangement of **63** to **64** was undertaken.

Synthesis - The substituted acetophenone *O*-(diethyl phosphoryl)oximes were prepared as previously described[10] except the reaction and work-up procedures were conducted at temperatures below 10°C. The pmr spectrum of **63** prepared in this manner gave a singlet for the acetophenone methyl at $\delta$ 2.29 and a singlet for ring methoxy at $\delta$ 3.75. Hydrolysis of **63** in aqueous ethanolic sodium hydroxide gave *p*-methoxyacetophenone oxime as the sole organosoluble product. Synthesis of **63** by the method previously used[10] afforded a product whose pmr spectrum showed two different singlets for C-methyl protons at $\delta$ 2.03 and $\delta$ 2.30, and two singlets for methoxy protons at $\delta$ 3.66 and $\delta$ 3.75. Alkaline hydrolysis of this material gave a mixture of p-methoxy-acetophenone and p-methoxy-acetanilide as the organosoluble products, indicating that the compound previously examined as 63 for toxicity and anticholinesterase activity was actually a mixture of 63 and 64.

For the preparation of 64, the general procedure of Atherton *et al*.[12] was used by reacting equimolar amounts of silver diethyl phosphate and p-methoxyphenylacetimidoyl chloride. Structures were confirmed by pmr spectroscopy.

Thermal Rearrangement - Pmr analysis was the principal method used for the study of the rearrangement of 63. The rate of rearrangement was monitored by estimating the appearance of the C-methyl proton of the imidoyl phosphate at $\delta$ 2.03 relative to the disappearance of the acetophenone C-methyl protons at $\delta$ 2.30.

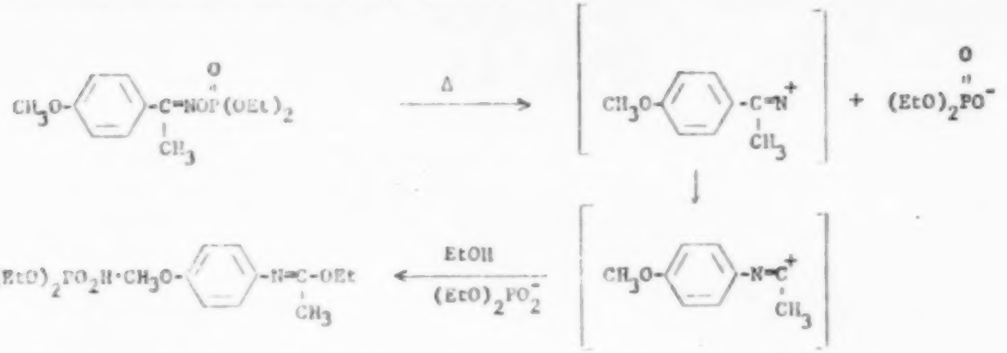
When a sample of pure 63 was heated in a sealed tube under nitrogen at 90° for 15 hr, the singlets at $\delta$ 2.30 and $\delta$ 3.75 disappeared and were replaced by singlets at $\delta$ 2.06 and $\delta$ 2.11 for methyl protons and a single peak for methoxy absorption at $\delta$ 3.68. Alkaline hydrolysis of this material produced only p-methoxyacetanilide. Similar results were obtained when pure samples of the O-(diethyl phosphoryl)oximes of acetophenone, p-methyl-, p-fluoro-, p-bromo-, and p-chloracetophenone were treated under the same conditions. In all cases the substituted acetanilide was the only product isolated after hydrolysis with sodium hydroxide.

Further, passage of pure 63 through the falling-film molecular distillation apparatus at a temperature range of 82-105°C (0.08 mm) gave successive fractions whose pmr spectra (carbon tetrachloride) were virtually identical with methyl absorptions at $\delta$ 2.03, $\delta$ 2.08 and $\delta$ 2.30 and methoxy absorptions at $\delta$ 3.70 and $\delta$ 3.80. Alkaline hydrolysis of each fraction produced a mixture of p-methoxyacetophenone oxime and p-methoxyacetanilide.

Although purification of diethyl N-p-methoxyphenylacetimidoyl phosphate (64) prepared from silver diethyl phosphate and p-methoxyphenylacetimidoyl chloride was not possible owing to the instability of this compound, pmr spectrum of the impure material gave methyl absorptions at $\delta$ 2.06 and $\delta$ 2.11, and methoxy absorption at $\delta$ 3.73 (in d-chloroform). This material decomposed upon standing to precipitate p-methoxyacetanilide. The presence of C-methyl proton absorptions at $\delta$ 2.06 and $\delta$ 2.11 provides evidence that the product obtained from the thermal rearrangement of 63 is 64.

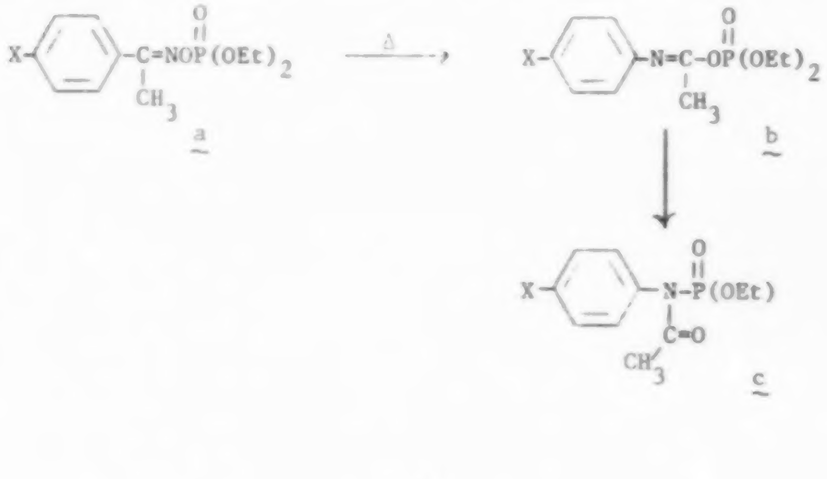
From the data for the rate of thermal rearrangement of 63 to 64 at 82°, 92°, and 105°C, first-order rate constants of 0.054, 0.090, and 0.297 (k, $\text{min}^{-1}$), respectively, were calculated. An Arrhenius plot gave a value of 8.2 Kcal/mole for the activation energy, $E_a$.

The thermal rearrangement of 63 was examined also in the presence of a nucleophilic solvent, ethyl alcohol, at 70°C. The reaction was examined spectrophotometrically every 10 min for 150 min and the spectral changes are shown in Fig. 5. 63 in ethyl alcohol shows an absorption maximum at 265 nm which upon standing at 70° gradually disappeared with the simultaneous appearance of a new maximum at 237 nm. The reaction was essentially complete after 150 min since no further change in the spectrum occurred after another 2.5 hr. The spectrum at this time was identical to the spectrum obtained from equimolar amounts of ethyl N-p-methoxyphenylacetimidate and diethyl phosphoric acid in ethyl alcohol. The results indicate that rearrangement of 63 and solvolysis of the intermediate carbonium ion to give ethyl N-p-methoxyphenylacetimidate takes place according to the following equations.



In the absence of nucleophilic solvent the imidoyl phosphate 64 is formed.

Diethyl N-Acetyl-N-Substituted-Phenylphosphoramidates - During the course of this study on the thermal rearrangement of substituted acetophenone O-(diethyl phosphoryl)oximes to the substituted N-phenylacetimidoyl diethyl phosphate, it occurred to us that further rearrangement of the latter to diethyl N-acetyl-N-phenylphosphoramidates was possible according to the equation below. The



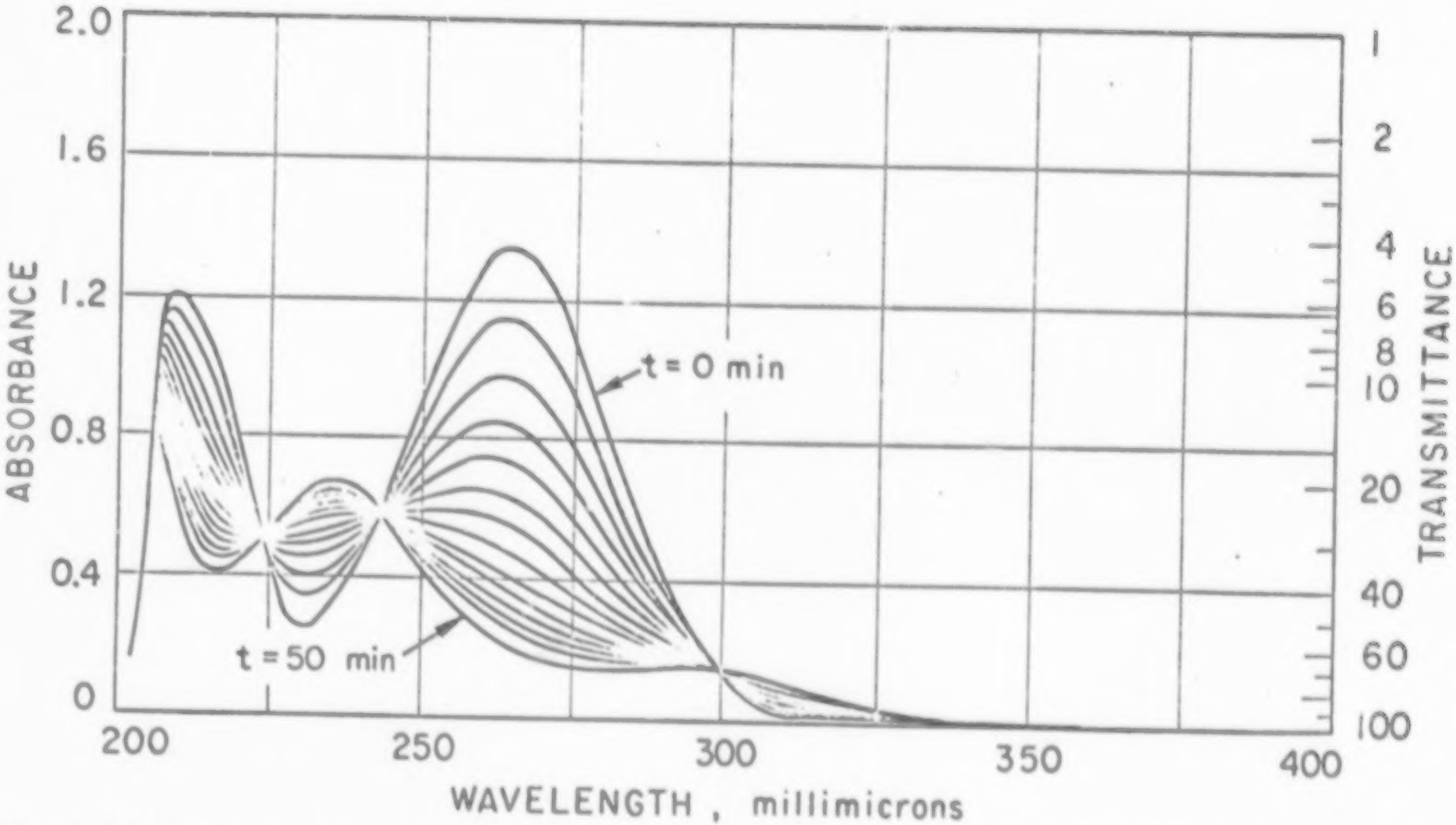


Figure 5. Absorption spectra showing the rearrangement and solvolysis of p-methoxyacetophenone O-(diethyl phosphoryl)oxime in absolute ethanol at 70°.

phosphoramidate **c** having the same empirical formula as **a** or **b** would give the same elemental analysis and alkaline hydrolysis conceivably would give acetanilide by cleavage of the P-N bond. In order to check out this possibility, several key diethyl *N*-acetyl-*N*-phenylphosphoramidates were prepared and compared by pmr and infrared analysis with the products obtained from the thermal rearrangement of the oxime phosphate. The methyl protons in all cases appeared as singlets at $\delta$ 1.98-2.00 and this coupled with infrared spectral data indicated that **c** was not obtained upon heating **a**.

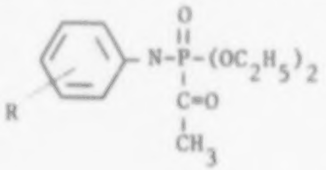
Examination of diethyl *N*-acetyl-*N*-phenylphosphoramidate and *N*-acetyl-*N*-*p*-methoxyphenylphosphoramidate, however, showed that each was moderately toxic to house flies and effective as an anticholinesterase. The unexpected biological activity, therefore, stimulated further examination of these compounds and a series of diethyl *N*-acetyl-*N*-phenylphosphoramidates were synthesized and assessed for insecticidal and anticholinesterase activity. Biological data for the various compounds are given in Table 4.

The data shows that all of the *N*-acetylphosphoramidates listed in the table are moderately good inhibitors of house fly-head cholinesterase with bimolecular inhibition constants in the region of $10^4$ $\underline{M}^{-1}$ $\text{min}^{-1}$. The most potent inhibitor in the series was the unsubstituted amidate (**65**) which had a $k_i$ value of $5.1 \times 10^4$ $\underline{M}^{-1}$ $\text{min}^{-1}$. Examination of the inhibition data showed no obvious correlation with any of the reactivity parameters. Compounds containing electron-withdrawing substituents such as the halogen in **70**, **71**, **72**, **73** and **74** were all less effective anticholinesterases than **65**. Compound **69** which contains the *p*-thiomethyl moiety (sigma constant of zero) was almost equal to **65** in activity.

The toxicity data show that several of the diethyl *N*-acetyl-*N*-phenylphosphoramidates were toxic to the house fly and toxicity was enhanced by the use of piperonyl butoxide. As in the case of anticholinesterase activity, **65** was most effective with an LD50 value of 60.5 μg/g. Again, no obvious correlation between toxicity and reactivity parameters was evident although it is noteworthy that the two poorest anticholinesterases (**71** and **74**) were least toxic to house flies.

Conclusion - The results show that *O*-(diethyl phosphoryl)oxime of ring-substituted acetophenones, particularly those with electron-donating substituents, are thermally unstable and readily undergo rearrangement to give the substituted *N*-phenylacetimidoyl diethyl phosphate. The rearrangement product is extremely unstable to traces of moisture and rapidly decomposes to the substituted acetanilide and diethyl phosphoric acid. *p*-Methoxyacetophenone *O*-(diethyl phosphoryl)oxime (**63**) may be prepared free from any rearranged material by carefully avoiding heat during any part of the reaction or work-up. Heat also must be avoided in preparing the corresponding

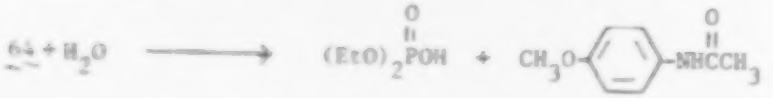
Table 4. TOXICOLOGICAL PROPERTIES OF O,O-DIETHYL N-ACETYL-N-PHENYLPHOSPHORAMIDATES

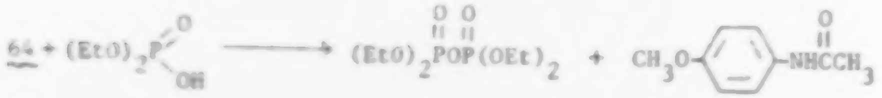


| Compound | $I_{50}$ M | $k_1$ $M^{-1}$ $min^{-1}$ | *Musca domestica*<br>$LD_{50}$ µg/g | *Musca domestica*<br>+ P.B. (1:5)<br>$LD_{50}$ µg/g | *Culex pipiens*<br>$LC_{50}$ ppm |
|---|---|---|---|---|---|
| 65 H | $2.40 \times 10^{-7}$ | $5.1 \times 10^{4}$ | 60.5 | 35 | 4.7 |
| 66 p-$CH_3S$ | $3.40 \times 10^{-7}$ | $4.8 \times 10^{4}$ | 425 | 56.5 | >10 |
| 67 p-$CH_3$ | $1.40 \times 10^{-6}$ | $3.2 \times 10^{4}$ | 100 | 55 | >10 |
| 68 p-$CH_3O$ | $9.40 \times 10^{-7}$ | $2.5 \times 10^{4}$ | 140 | 59 | >10 |
| 69 o-$CH_3O$ | $1.20 \times 10^{-6}$ | $3.8 \times 10^{4}$ | 420 | 180 | >10 |
| 70 p-Cl | $1.32 \times 10^{-6}$ | $2.9 \times 10^{4}$ | 180 | 60 | >10 |
| 71 o-Cl | $7.20 \times 10^{-5}$ | $3.3 \times 10^{3}$ | >500 | 245 | >10 |
| 72 m-Cl | $1.15 \times 10^{-6}$ | $1.9 \times 10^{4}$ | 180 | 47 | >10 |
| 73 p-Br | $2.04 \times 10^{-6}$ | $1.5 \times 10^{4}$ | 200 | 75 | >10 |
| 74 p-F | $1.60 \times 10^{-6}$ | $1.4 \times 10^{4}$ | 145 | 60 | >10 |
| 75 o-iPr | $4.40 \times 10^{-5}$ | $8.3 \times 10^{3}$ | >500 | 170 | >10 |

*p*-methyl and unsubstituted analogs. The rather low $E_a$ value (8.2 Kcal) for the thermal rearrangement of **63** provides a measure for the instability of *O*-(diethyl phosphoryl)oximes of ring-substituted acetophenones containing electron-donating substituents. Overall, the results show that care must be taken in avoiding heat when compounds of this type are prepared.

The compound reported[10] previously as **63** was unusually potent as an inhibitor of fly-head cholinesterase, the molar concentration to give 50% inhibition of enzyme ($I_{50}$) was 7.2 X $10^{-9}$ and moderately toxic to house flies, $LD_{50}$ 27 µg/g/fly. However, **63** prepared without application of any heat, was virtually nontoxic to the house fly ($LD_{50} > 500$ µg/g) and was about 500-fold less effective in inhibiting fly-head cholinesterase ($I_{50}$ 3.45 X $10^{-6}$). Thus, it appears that the strong anticholinesterase activity and house fly toxicity reported earlier for **63** was caused by the rearranged product **64** rather than **63**. Although **64** undoubtedly is formed when **63** is heated, it is possible also that tetraethyl pyrophosphate (TEPP) also may be formed in the presence of small amounts of moisture according to the equations below:



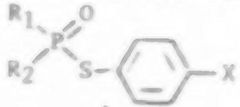


The reaction between dialkyl imidoyl phosphate and dialkyl phosphoric acid to give the corresponding pyrophosphate has been established by Atherton *et al.*[12]. Therefore, the possibility exists that TEPP also may be contributing to the toxicological properties of impure **63**.

Comparison of pmr and infrared spectra showed that diethyl *N*-acetyl-*N*-phenylphosphoramidates are not produced from the thermal rearrangement of the corresponding *O*-(diethyl phosphoryl)oximes of substituted acetophenone. The *N*-acetyl-*N*-phenylphosphoramidates (**65**-**75**), however, were of considerable interest owing to their unexpected anticholinesterase activity and toxicity to insects. These compounds are unusual in that the most likely bond to break when cholinesterase enzyme is phosphorylated in the inhibition process is the P-N linkage and apparently the acetanilide anion is the leaving group. Compounds of this type deserve further examination as possible insecticides.

## 4. Toxicologocal and Hydrolytic Properties of S-Aryl Phosphoramidothioates

Introduction - Previous studies[13] concerning the relationship between structure, reactivity and insecticidal activity of O-alkyl S-alkyl phosphoramidothioates revealed that several of the compounds were exceptionally toxic to the housefly, although they were relatively weak inhibitors of fly-head AchE. One of these compounds, Monitor or O,S-dimethyl phosphoramidothioate has been developed as a useful insecticide. The outstanding insecticidal properties of compounds of this type, combined with the limited amount of information available on the chemistry and mode of action of phosphoramidothioate esters, prompted us to extend our investigations to include phosphoramidothioates containing aryl moieties. This section is concerned with the chemical, biochemical, and toxicological properties of O-ethyl S-aryl phosphoramidothioates and related esters of the general structure indicated (A) where $R_1$ is $NH_2$, $NHCH_3$ or $N(CH_3)_2$, and $R_2$ is $C_2H_5$ or $OC_2H_5$.



A

Synthesis - O,O-Diethyl substituted S-phenyl phosphorothioates were prepared by established procedures from the condensation of the substituted sodium benzenethiolate with diethyl phosphorochloridate or by the reaction of triethyl phosphite with the appropriate benzenesulfonyl chloride. The O-ethyl S-phenyl phosphorochloridothioates were prepared from the corresponding O,O-diethyl S-phenyl phosphorothioates by the action of phosphorus pentachloride according to the procedure used to convert phosphonates to phosphonochloridates. Evidently, this procedure has not been applied to phosphate or phosphonate esters containing an S-phenyl moiety but the reaction was quite successful, as yields up to 64% were obtained. The O-ethyl S-phenyl phosphoramidothioates were prepared from the corresponding chloridothioates by reaction with ammonia or the appropriate amine.

Hydrolysis - Data for the alkaline hydrolysis and toxicological properties of the various S-aryl phosphoramidothioates and phosphonamidothioates are given in Table 5. Alkaline hydrolysis rates were determined spectrophotometrically by estimating directly the amount of arylthiolate ion formed under pseudo-first-order conditions of excess sodium hydroxide. Excellent first-order plots were obtained in all cases, indicating that hydrolysis occurred essentially by P-S bond cleavage.

For the various O-ethyl substituted S-phenyl phosphoramidothioates in Table 5, a linear relationship was observed between the logarithm of the calculated second-order hydrolysis constants, $k_2$, and Hammett's $\sigma$ constants derived from substituted benzoid acids. From the line fitting the data (correlation coefficient $r = 0.99$), the value of 1.27 for the reaction constant, $\rho$, was calculated. This value is very similar to the $\rho$ values of 1.03 and 1.32 calculated for the hydrolysis of O,O-diethyl substituted S-phenyl phosphorothioates[14] and diethyl substituted phenyl phosphates,[15] respectively. The close similarity of $\rho$ values in these cases suggests that the hydrolytic reaction in these three cases takes place by a common mechanism.

Comparison of 78, 84, and 85, compounds of identical structure except for the number of methyl groups on the amido nitrogen, shows that sequential replacement of hydrogen by a methyl group results in marked increase in hydrolytic stability, e.g., $k_2$ ($M^{-1}$ min$^{-1}$) is 56.0 for 78, 1.1 for 84, and $4.97 \times 10^{-3}$ for 85. Similarly, the $k_2$ value of 0.63 for the N-methylphosphonamidothioate, 86, is approximately 11-fold greater than $k_2$ of 0.050 for the N,N-dimethyl analog, 87. The difference in this case, however, is much smaller than the 200-fold difference between the analogous phosphoramidothioates, 84 and 85.

Values for the energy ($E_a$) and entropy ($\Delta S^{\ddagger}$) of activation for 78, 84, 85, 86, and 87 are given in Table 5. The data show that the range in $E_a$ for the five compounds is small (14.0-15.7 kcal/mol). In comparison, the change in $\Delta S^{\ddagger}$ is much more substantial, becoming uniformly more negative with each replacement of hydrogen by a methyl group. On this basis it appears that the decrease in $k_2$ with nitrogen substitution is caused more by steric restraints than by electronic effects.

The rate constant, $k_2$, for the hydrolysis of 84, a phosphoramidothioate, is almost two-fold larger than $k_2$ for the corresponding phosphonamidothioate, 86. The significant but faster rate of hydrolysis of 84 compared to 86 was unexpected, since phosphonate esters generally are less stable to alkali than phosphate esters. From the values of $E_a$ and $\Delta S^{\ddagger}$ for 84 and 86, the free energy of activation $\Delta F^{\ddagger}$ was calculated to be 17.8 and 18.1 kcal/mol, respectively, values which are consistent with the second-order hydrolysis constants ($k_2$).

Cholinesterase Inhibition and Toxicity - The data in Table 5 for anticholinesterase activity show that all of the primary phosphoramidothioates (76-83) are moderately strong inhibitors of fly-head cholinesterase with $k_1$ values ($M^{-1}$ min$^{-1}$) ranging from approximately $1 \times 10^5$ to $5.9 \times 10^5$. Attempts to correlate rates of cholinesterase inhibition with $k_2$ or any of the free energy parameters for ring substituents ($\sigma$, $\pi$, $\pi^2$), or combination of these parameters, proved to be unfruitful. The failure to obtain a suitable relationship between $k_1$ and the various parameters was disappointing, since an excellent correlation between $k_1$ and a combination of $\pi$ and hydrolysis rates previously was demonstrated[16] for a series of methyl substituted phenyl *N*-methylphosphoramidates.

Progressive substitution of the hydrogens on the amido nitrogen by a methyl group resulted in expected decrease in anticholinesterase activity. Within this limited series of compounds a reasonably good linear relationship was obtained between log $k_1$ and log $k_2$, indicating that anticholinesterase activity is primarily a function of the reactivity of the ester.

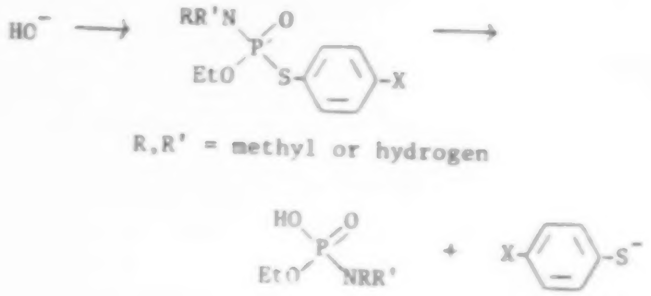
The primary phosphoramidothioates in Table 5 showed wide variability in their toxicity to houseflies compared to their activity as anticholinesterases. In general, these esters were substantially less toxic than the primary *O*,*S*-dialkyl phosphoramidothioates[13]. For example, *O*,*S*-dimethyl phosphoramidothioate (Monitor) with an $LD_{50}$ to houseflies of 1.3 μg/g is approximately six-fold more effective than *O*-ethyl *S*-*p*-chlorophenyl phosphoramidothioate (**78**), the most toxic compound in this series. Compared to Monitor ($k_1 = 9.2 \times 10^2$ $M^{-1}$ $\text{min}^{-1}$), however, **78** is about 450-fold more effective in inhibiting fly-head cholinesterase. It appears that anticholinesterase activity is not a useful guide for the prediction of the insecticidal activity of phosphoramidothioates.

As in the case of cholinesterase inhibition, progressive replacement of hydrogens on the amido nitrogen by methyl groups resulted in a decrease in housefly toxicity. For example, the housefly $LD_{50}$ (μg/g) of the primary amidate (**78**) was 8.2, the monomethylamidate (**84**) was 13, and dimethylamidate was 83. The effect of sequential methyl substitution on the reduction of toxicity, however, is much less in comparison to the *O*,*S*-dialkyl phosphoramidothioate analogs, e.g., the toxicity of the *N*-methyl derivative of Monitor to houseflies ($LD_{50}$, μg/g) was 49 and of *N*,*N*-dimethyl was >500.

The virtual absence of toxicity demonstrated by *S*-*p*-chlorophenyl *N*-methyl-*P*-ethylphosphonamidothioate (86) toward houseflies ($LD_{50}$ >500 μg/g) was surprising since measurable toxicity was found for the corresponding *N*,*N*-dimethyl analog ($LD_{50}$ 75 μg/g). Unfortunately, comparison with the primary phosphonamidothioate was not possible since all attempts to synthesize this compound were unsuccessful. Further, the corresponding *N*-methylphosphoramidothioate analog (**84**) with $k_1$ and $k_2$ values similar to those of **86** also was quite toxic to flies. The toxicity of **86**, however, was pronouncedly synergized when applied together with 5:1 parts piperonyl butoxide ($LD_{50}$ 17 μg/g). The level of synergism of this magnitude, although quite common for methylcarbamate esters, is unusual for organophosphorus esters and suggests that **86**, for some reason, is peculiarly susceptible to oxidative detoxication.

In limited studies using the white mouse, an $LD_{50}$ value of 5-8 mg/kg was obtained for **78**. This compound, therefore, is approximately of equal toxicity to houseflies and mice.

Conclusion - The excellent correlation obtained between $\sigma$ and log $k_2$ for the primary phosphoramidothioates (76-83), combined with the similarity in the magnitude of $\rho$ calculated for this series and for substituted phenyl diethyl phosphates and phosphorothioates, indicates that substituent effects are transmitted from the ring to the phosphorus atom with equal facility through sulfur and oxygen. The alkaline hydrolysis of diethyl phenyl phosphates and diethyl S-phenyl phosphorothioates undoubtedly takes place by nucleophilic attack of hydroxide ion on the phosphoryl center. In light of the similarity in $\rho$ values for the three series of esters, it appears that S-phenyl phosphoramidothioates also hydrolyze by a direct displacement reaction on phosphorus, as shown below.



R,R' = methyl or hydrogen

The values for $E_a$ and $\Delta S^{\ddagger}$ for 78, 84 and 85, and for 86 and 87 also are consistent with a direct displacement mechanism. As indicated earlier, there is a small but significant increase in $E_a$, accompanied by a larger but uniform decrease in $\Delta S^{\ddagger}$ with each replacement of hydrogen by a methyl group. The decrease in $k_2$, therefore, can be accounted for primarily on the basis of polar and steric effects created by each succeeding methyl group.

It is apparent from the toxicological data that the insecticidal activity of the various phosphoramidothioates and phosphonamidothioates in Table 5 can not be anticipated from the structure nor from any of the reactivity parameters. S-Aryl phosphoramidothioates generally are less toxic to insects than the simple S-alkyl phosphoramidothioates, although they are substantially more effective as anticholinesterases. However, because of the unpredictable variability in their insecticidal activity, other esters of related structure deserve to be examined.

## 5. Physical and Chemical Basis for Systemic Movement of Organophosphorus Esters in Plants

Introduction - Relatively little information is available on the relationship between chemical structure and the ability of the

Table 5. TOXICOLOGICAL AND HYDROLYTIC PROPERTIES OF S-PHENYL PHOSPHORAMIDOTHIOATES OF GENERAL STRUCTURE A

|  | $R_1$ | $R_2$ | X | Fly-head ChE $k_i \times 10^{-5}$ ($M^{-1}$ $min^{-1}$) 37.5° | Musca domestica $LD_{50}$ (µg/g) | Culex pipiens $LC_{50}$ (ppm) | 2nd order $k_2$ ($M^{-1}$ $min^{-1}$) 29.5° | $E_a$ (Kcal/mole) | $\Delta S^{\ddagger}$ (e.u.) |
|---|---|---|---|---|---|---|---|---|---|
| 76 | $NH_2$ | $OC_2H_5$ | H | 2.10 | 22.0 | 0.096 | 25.8 |  |  |
| 77 | $NH_2$ | $OC_2H_5$ | F | 0.961 | 27.0 | 0.012 | 31.6 |  |  |
| 78 | $NH_2$ | $OC_2H_5$ | Cl | 4.32 | 8.2 | 0.15 | 56.0 | +14.0 | -6.3 |
| 79 | $NH_2$ | $OC_2H_5$ | Br | 5.60 | 11.0 | 0.024 | 55.7 |  |  |
| 80 | $NH_2$ | $OC_2H_5$ | $CH_3$ | 5.27 | 12.5 | 0.044 | 17.3 |  |  |
| 81 | $NH_2$ | $OC_2H_5$ | $C_2H_5$ | 5.86 | 55.0 | 0.34 | 17.1 |  |  |
| 82 | $NH_2$ | $OC_2H_5$ | $(CH_3)_2CH$ | 4.37 | 32.0 | >1.0 | 17.4 |  |  |
| 83 | $NH_2$ | $OC_2H_5$ | $(CH_3)_3C$ | 2.60 | 100.0 | >1.0 | 16.9 |  |  |
| 84 | $NHCH_5$ | $OC_2H_5$ | Cl | 0.262 | 13.0 | 0.92 | 1.10 | +14.4 | -12.8 |
| 85 | $N(CH_3)_2$ | $OC_2H_5$ | Cl | 0.0924 | 83.0 | >1.0 | 0.00497 | +15.6 | -19.6 |
| 86 | $NHCH_3$ | $C_2H_5$ | Cl | 0.545 | >500 (17)[a] | 0.92 | 0.632 | +15.5 | -10.2 |
| 87 | $N(CH_3)_2$ | $C_2H_5$ | Cl | b | 75.0 | 0.93 | 0.0551 | +15.7 | -14.3 |

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[a] With piperonyl butoxide 5:1 (w/w).

[b] A linear pseudo-first-order inhibition rate could not be obtained.

compound to be translocated in a plant. Since a wide variety of organic insecticides of different physical and chemical properties have been demonstrated to move systemically in plants, the question, what are the critical factors which govern systemic in movement, has often been raised. The following discussion presents results of a systematic analysis of the relation between systemic movement in the cotton plant of a series of phosphor midothioate esters and their octanol-water partitioning properties. These compounds were selected because of the known systemic insecticidal activity and stability in plants of *O*,*S*-dimethyl phosphoramidothioate (Monitor), the parent compound of the series.

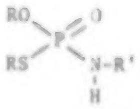
*Synthesis and Method of Analysis* - *O*,*S*-Dialkyl phosphoramidothioates were prepared in an earlier study.[13] The *O*,*O*-dimethyl *N*-alkylphosphoramidothioates were prepared according to conventional methods by reaction of the desired amine with dimethyl phosphorochloridothionate in anhydrous ether. Liquid products were purified by distillation at reduced pressure and solid products by recrystallization from n-hexane. The *O*,*O*-dimethyl *N*-alkylphosphoramidothioates were isomerized to *O*,*S*-dimethyl *N*-alkylphosphoramidothioates by heating the thionate in a several fold excess of methyl iodide.[13]

Octanol-water partition coefficients were determined according to Fujita et al.[17] Analysis of each phase was conducted by gas-liquid chromatography (glc) or UV spectrophotometry. Glc also was the principal means for the estimation of translocated materials in leaf tissue. Excised cotton leaves placed in water were treated on the petiole 2" below the leaf blade. The amount of material moved from the petiole surface into the blade was analyzed after appropriate time intervals.

*Octanol-Water Partition* - Relevant data concerning octanol-water partitioning properties of the various phosphoramidothioate esters and miscellaneious organophosphorus insecticides are presented in Table 6. For the *O*,*S*-dimethyl *N*-alkylphosphoramidothioate analogs (**88**-**96**) a gradual increase in the octanol-water partition coefficient (P) was observed as the *N*-alkyl chain size was increased, although there appeared to be an abrupt increase between the *N*-ethyl and *N*-propyl derivatives (compare **89** and **90**). Branching in the alkyl group of the *N*-butyl analogs (**91** and **94**) did not result in any significant change in partition coefficients. The partition coefficients for the limited number of *O*,*S*-dialkyl phosphoramidothioates (**97**-**99**), as expected, also increased with lengthening of the alkyl chain on oxygen and sulfur but the relative increase appeared to be slightly greater than with the *N*-alkyl derivatives.

Water solubility, in the absence of significant inductive or polar effects, should be inversely related to octanol-water partition coefficients. As expected, the water solubilities of the analogous series of *O*,*S*-dimethyl *N*-alkylphosphoramidothioates decreased as

Table 6. PHYSICAL AND CHEMICAL PROPERTIES OF O,S-DIALKYL N-ALKYLPHOSPHORAMIDOTHIOATES AND OTHER ORGANOPHOSPHORUS INSECTICIDES



| | R | R' | % Distribution octanol phase | % Distribution water phase | Partition coefficient (P) | Log P | - | Water solubility (mg/ml, 25°C) | $E_s$ |
|---|---|---|---|---|---|---|---|---|---|
| 88 | $CH_3$ | $CH_3$ | 46.22 | 53.78 | 0.86 | -0.07 | 0.59 | >1000.00 | -1.24 |
| 89 | $CH_3$ | $C_2H_5$ | 54.21 | 45.79 | 1.18 | 0.07 | 0.73 | >1000.00 | -1.31 |
| 90 | $CH_3$ | n-$C_3H_7$ | 81.75 | 18.25 | 4.48 | 0.65 | 1.31 | 176.10 | -1.60 |
| 91 | $CH_3$ | n-$C_4H_9$ | 89.71 | 10.29 | 8.71 | 0.94 | 1.60 | 58.61 | -1.63 |
| 92 | $CH_3$ | s-$C_4H_9$ | 89.85 | 10.15 | 8.85 | 0.95 | 1.61 | 67.78 | -2.37 |
| 93 | $CH_3$ | iso-$C_4H_9$ | 90.37 | 9.63 | 9.39 | 0.97 | 1.63 | - | -2.17 |
| 94 | $CH_3$ | t-$C_4H_9$ | 90.02 | 9.98 | 9.03 | 0.96 | 1.62 | 78.88 | -2.78 |
| 95 | $CH_3$ | n-$C_6H_{13}$ | 92.79 | 7.21 | 12.87 | 1.11 | 1.77 | 4.92 | -1.60 |
| 96 | $CH_3$ | n-$C_8H_{17}$ | 97.00 | 3.00 | 32.33 | 1.51 | 2.17 | 0.36 | -1.57 |
| 97 | $CH_3$ | H | 18.00 | 82.00 | 0.22 | -0.66 | 0.00 | >1000.00 | - |
| 98 | $C_2H_5$ | H | 58.50 | 41.50 | 1.41 | 0.15 | | >200.00 | - |
| 99 | n-$C_3H_7$ | H | 94.50 | 5.50 | 17.20 | 1.23 | - | - | - |
| 100 | demeton thiol | | 98.85 | 1.15 | 85.96 | 1.93 | - | 2.00[a] | - |
| 101 | dimethoate | | 76.25 | 23.75 | 3.21 | 0.50 | - | 25.00[a] | - |
| 102 | methyl paraoxon | | 95.00 | 5.00 | 19.00 | 1.28 | - | 6.43 | - |

Table 6 continued.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 103 | paraoxon | 97.50 | 2.50 | 38.84 | 1.59 | - | 2.4 | - |
| 104 | naled | 96.00 | 4.00 | 24.00 | 1.38 | - | - | - |

the length of the alkyl chain was increased and the n-octyl analog (96) was found to be the least soluble compound in the series.

The octanol-water partition coefficients of the other organophosphorus esters were in the order: demeton thiol > paraoxon > naled > methyl paraoxon > dimethoate. The literature values for the water solubilities of these compounds were found to be in the reverse order, except for naled for which a water solubility value was not available.

Systemic Movement - Data for the movement of the various organophosphorus esters from the leaf petiole surface into the leaf blade are given in Table 7. From the relative amounts translocated at the indicated time intervals, the values for the rate of translocation ($k_{tr}$) were calculated. Examination of the data for the O,S-dimethyl N-n-alkylphosphoramidothioate series (88-91, 95, 96) reveals the existence of a parabolic relationship between the rate of translocation and octanol-water partition coefficients and optimum rate was found with the N-propyl derivative (90). The parabolic relationship which is obtained is shown graphically in Figure 6 where log $k_{tr}$ is plotted against log P. A similar plot was obtained when log $k_{tr}$ was plotted against $\pi$. This is to be expected since for an analogous series of compounds $\pi$ is directly proportional to log P.

The values of $k_{tr}$ and $\pi$ for the O,S-dimethyl N-n-alkylphosphoramidothioates were subjected to multiple regression analysis. By linearly combining $\pi$ and $\pi^2$ terms the following regression equation 13 was obtained (r = correlation coefficient, n = number of compounds).

$$\text{Log } k_{tr} = -0.753 + 2.318\pi - 0.974\pi^2 \qquad (13)$$

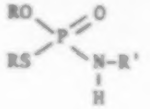
$$r = 0.999 \qquad n = 6$$

The excellent value for the correlation coefficient (r) indicates good fit of the data to the equation.

For the limited number of O,S-dialkyl phosphoramidothioates examined (97, 98 and 99), a similar parabolic relationship, as with the N-n-alkyl derivatives, was observed between $k_{tr}$ and $\pi$ (or log P). The replacement of the methyl groups in 97 with ethyl (98) resulted in increased lipophilicity as well as in systemic movement. However, substitution with propyl evidently resulted in excessive lipophilicity and $k_{tr}$ for the di-n-propyl derivative (99) was substantially less than that for 98.

Although the O,S-dialkyl phosphoramidothioates and O,S-dimethyl N-n-alkylphosphoramidothioates are structurally closely related to each other, it appears that the two series of compounds follow a

Table 7. TRANSLOCATION DATA FOR THE VARIOUS O,S-DIALKYL N-ALKYLPHOSPHORAMIDOTHIOATES AND OTHER ORGANOPHOSPHORUS INSECTICIDES



| | | | % of Applied amount moved | | | | Rate of movement in petiole | $r^2$ for slope |
|---|---|---|---|---|---|---|---|---|
| | R | R' | Time (hrs) 2 | 4 | 8 | 12 | ($k_{tr}$) | |
| 88 | $CH_3$ | $CH_3$ | 3.31 | 5.59 | 15.97 | 21.35 | 1.97 | 0.967 |
| 89 | $CH_3$ | $C_2H_5$ | 3.29 | 8.63 | 21.37 | 29.23 | 2.57 | 0.981 |
| 90 | $CH_3$ | n-$C_3H_7$ | 3.12 | 11.13 | 25.70 | 40.81 | 3.71 | 0.999 |
| 91 | $CH_3$ | n-$C_4H_9$ | 7.04 | 14.00 | 30.90 | 39.90 | 3.24 | 0.970 |
| 92 | $CH_3$ | s-$C_4H_9$ | 3.21 | 10.21 | 22.60 | 27.30 | 2.13 | 0.937 |
| 93 | $CH_3$ | iso-$C_4H_9$ | 1.80 | 7.50 | 13.85 | 15.10 | 0.95 | 0.869 |
| 94 | $CH_3$ | t-$C_4H_9$ | 0.12 | 0.28 | 0.67 | 0.68 | 0.05 | 0.768 |
| 95 | $CH_3$ | n-$C_6H_{13}$ | 0.22 | 7.26 | 15.30 | 23.28 | 2.00 | 0.999 |
| 96 | $CH_3$ | n-$C_8H_{17}$ | 0.68 | 0.91 | 3.18 | 4.80 | 0.48 | 0.991 |
| 97 | $CH_3$ | H | 3.85 | 9.47 | 22.00 | 37.13 | 3.45 | 0.998 |
| 98 | $C_2H_5$ | H | 2.44 | 12.90 | 25.90 | 59.10 | 5.77 | 0.940 |
| 99 | n-$C_3H_7$ | H | 2.25 | 10.86 | 33.15 | 42.55 | 3.92 | 0.944 |
| 100 | demeton thiol | | 11.45 | 18.12 | 21.82 | 16.83 | 2.61 | 1.000[a] |
| 101 | dimethoate | | 0.00 | 3.00 | 9.50 | 27.35 | 3.04 | 0.932 |
| 102 | methyl paraoxon | | 0.30 | 0.84 | 3.02 | 9.39 | 1.07 | 0.926 |
| 103 | paraoxon | | 2.39 | 8.25 | 20.86 | 34.90 | 3.33 | 0.999 |
| 104 | naled | | - | - | - | - | 1.77[b] | - |

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[a] Regression analysis for three points only. [b] Rate of movement for first 4 hrs only.

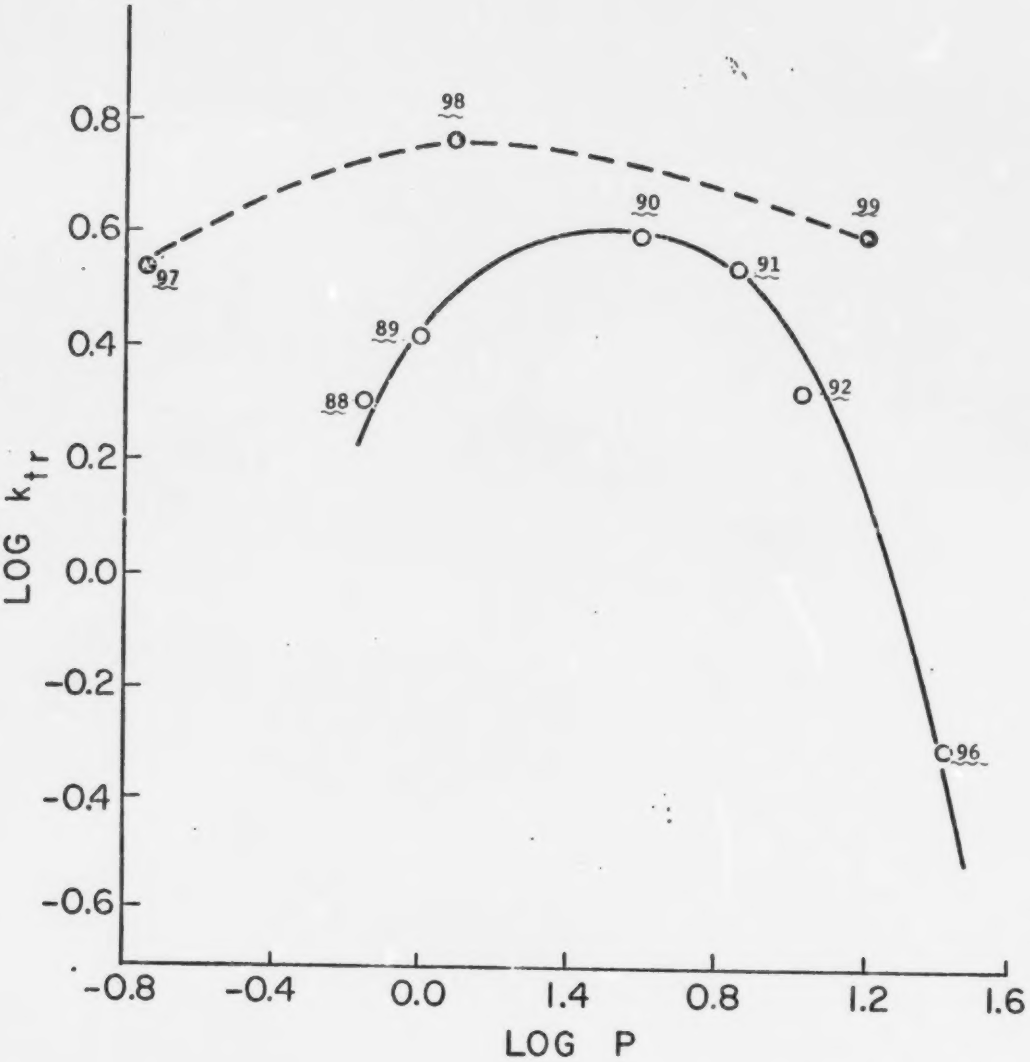

Fig. 6. Relationship between log $k_{tr}$ and log P for phosphoramidothioate esters; solid line is for O,S-dimethyl N-n-alkylphosphoramidothioates and dotted line is for O,S-dialkyl phosphoramidothioates.

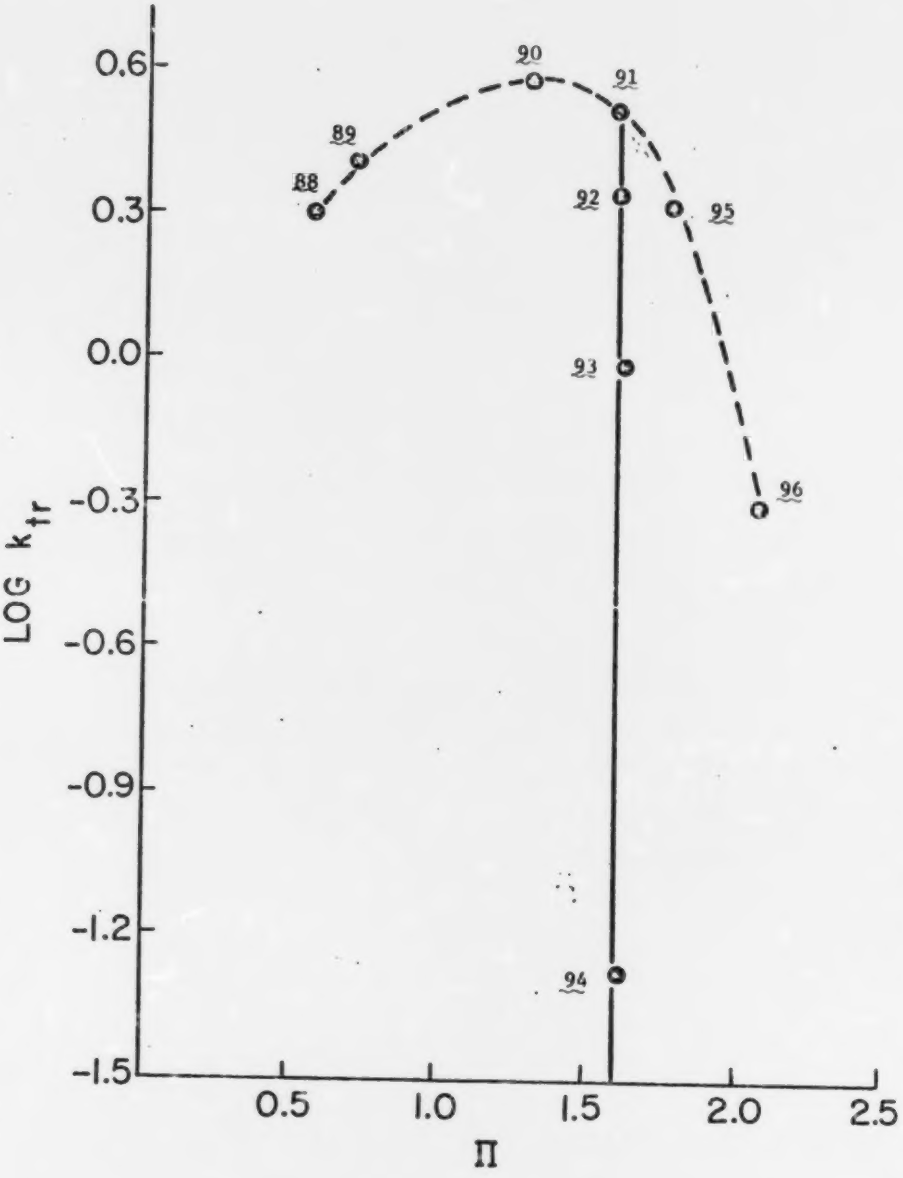

Fig. 7. Relationship between log $k_{tr}$ and $\pi$ for O,S-dimethyl N-alkylphosphoramidothioates; dotted line is for n-alkyl esters and solid line is for branched butyl esters.

separate parabolic relationship. This is evident from the dotted curve in Figure 6 and from regression analysis when data for **98** and **99** were included with those of the *N*-*n*-alkylphosphoramidothioates. In this case, regression equation 14 with a correlation coefficient of 0.844, was obtained.

$$\text{Log } k_{tr} = -0.736 + 2.401\pi - 0.992\pi^2 \qquad (14)$$

$$\underline{r} = 0.844 \qquad \underline{n} = 8$$

The significantly poorer correlation coefficient obtained compared to the *O*,*S*-dimethyl *N*-*n*-alkylphosphoramidothioate series (cf equation 13) indicates that the *N*-*n*-alkyl and unsubstituted phosphoramidothioates should be treated separately. Evidently, alkyl substitution on the amido nitrogen atom results in compounds with reduced rates of systemic movement in the cotton plant, even though individual *N*-substituted and unsubstituted phosphoramidothioates may have similar octanol-water partitioning properties.

Profound changes in systemic movement were observed when branching was introduced into the *N*-alkyl chain. For the various isomeric *N*-butyl derivatives (**91**, **92**, **93** and **94**), marked reduction in $k_{tr}$ values occurred with increase in branching and translocation was minimal with the *t*-butyl derivative (**94**). In spite of rather similar values of $\pi$ for the four different butyl isomers (1.60-1.63), there was approximately a 65-fold difference in $k_{tr}$ between the *n*-butyl and *t*-butyl derivatives with the values of the other two isomers ranging in between. The relation between $k_{tr}$ and for the four isomeric butyl derivatives is shown graphically in Figure 7 by the solid line. These results indicate that within the series of nine *O*,*S*-dimethyl *N*-alkylphosphoramidothioates, solvent partitioning properties alone, i.e., $\pi$ or log P, do not totally account for systemic movement.

The reduction in systemic movement with increase in branching in the *N*-butyl chain suggests that steric effects also should be considered in seeking correlation of the sytemic data. For the *O*,*S*-dimethyl *N*-alkylphosphoramidothioates (**88**-**96**), including the branched butyl derivatives, the following regression equation relating $k_{tr}$ and $\pi$ was obtained. The values for *F* (F-statistics) and standard error (*s*) are included.

$$\text{Log } k_{tr} = 0.307 + 0.273\pi - 0.267\pi^2 \qquad (15)$$

$$\underline{r} = 0.372;\ \underline{n} = 9;\ \underline{s} = 0.636;\ \underline{F} = 0.483$$

Use of Taft's steric substituent constant ($E_s$) alone in the analysis resulted in equation 16.

$$\text{Log } k_{tr} = 1.498 + 0.782E_s \qquad (16)$$

$\underline{r} = 0.680$; $\underline{n} = 9$; $\underline{s} = 0.465$; $\underline{F} = 6.033$

From the magnitude of the correlation coefficients for equation 15 and equation 16, evidently neither $E_s$ alone nor the combination of $\pi$ and $\pi^2$ alone are able to provide significant correlation with the translocation data. On the other hand, linear combination of $\pi$, $\pi^2$, and $E_s$ resulted in substantial improvement in correlation, in accordance with equation 17 below.

$$\text{Log } k_{tr} = -0.196 + 4.187\pi - 1.551\pi^2 + 1.209E_s \qquad (17)$$

$\underline{r} = 0.863$; $\underline{n} = 9$; $\underline{s} = 0.379$; $\underline{F} = 4.865$

The F-statistics show significant correlation at the 10% level. These results provide quantitative evidence that steric effects, in addition to solvent partitioning properties, are important in predicting systemic movement.

In addition to octanol-water partitioning properties, water solubilities of several representative phosphoramidothioates were determined (Table 6). The results indicate that, within the limits of the compounds examined, water solubility is not a critical factor in regulating the degree of systemic movement. For example, compounds 88 and 89 with solubilities of >1000 mg/ml were decidedly poorer in their ability to penetrate and move through the petiole than was 91, a compound of much lower water solubility. In contrast, the highly water soluble O,S-dimethyl phosphoramidothioate (97) translocated rapidly and the poorly soluble n-octyl derivative (96) quite slowly. Similarly, the systemic movement of the other organophosphorus esters (100-104) evidently was not related to their water solubilities.

Conclusion - The foregoing results clearly indicate that even within a relatively restricted series of compounds, systemic movement in the cotton plant is not directly correlated with any single chemical or physical parameter. This is not at all surprising since penetration and translocation of foreign materials in a plant are doubtlessly complex processes and a number of different factors must be involved.

The development of $\pi$ as a useful parameter originated from an attempt to correlate plant growth promoting properties with the structure of a series of substituted phenoxyacetic acids.[18] In this classic study $\pi$ was developed to account for substituent effects on the rate of penetration of an externally applied acid to the site of action. Penetration to the site of action was visualized as a random walk process involving multiple partitions between lipophilic and hydrophilic phases in the plant and the addition of $\pi$ to the regression equations provided highly significant improvement to the correlation. Therefore, the failure of $\pi$ (or log P) to correlate satisfactorily the rate of systemic movement of the phosphoramidothioates (compounds 88-95) was unexpected. Only in the limited case of the N-n-alkyl derivatives was satisfactory correlation obtained with $\pi$ and $\pi^2$ alone.

There is little doubt that steric effects play a significant role in determining the rate in which the N-alkylphosphoramidothioates penetrate and move in the cotton leaf petiole. The poor ability of the isobutyl (93) or t-butyl (94) derivatives to move systemically may be explained purely on the basis of the bulkiness of the branched butyl moieties which may impede the movement of the molecule through plant cuticle and membranes. Another possible explanation resides in hydration effects associated with the amido moiety. The effect of alkyl substituents in limiting the extent which water is solvated to amines is well known and the possibility remains that hydration of the amido moiety, or its absence owing to steric effects, may drastically affect rates of systemic movement. For example, in the case of 95, the bulky t-butyl group prevents solvation by water to occur, thus rendering the molecule more hydrophobic and increasing the possibility of retention by lipophilic phases in the plant. On the other hand, compounds without alkyl substituents on the amido proton probably are highly sovated by water and hydrophobic interaction with lipophilic plant constituents which reduces penetration and systemic movement is minimized. The greater than expected systemic movement of the O,S-dialkyl phosphoramidothioates (97-99) is consistent with this kind of interpretation.

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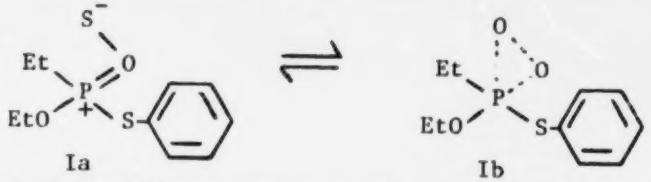
## F. STUDIES ON THE OXIDATIVE CONVERSION OF PS TO PO ESTERS

The oxidation of phosphorothionate (PS) to the corresponding phosphate (PO) ester in plants and animals is a well established phenomenon. Since the toxic action of phosphorothionate insecticides (generally poor inhibitors of the cholinesterase enzymes) is attributed to the anticholinesterase action of the related phosphate ester produced metabolically, the conversion of PS to PO esters is considered an activation reaction. PS to PO conversion, therefore, is an essential step in the intoxication of animals exposed to phosphorothionate esters. This part of the report is concerned with our studies on some of the fundamental aspects of this important reaction.

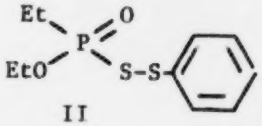
### 1. Oxidative Rearrangement of O-Ethyl S-Phenyl Ethylphosphonodithioate (Dyfonate)

Introduction - In an earlier communication McBain et al.[1] reported that an oxygenated product was obtained from *O*-ethyl *S*-phenyl ethylphosphonodithioate (Dyfonate) was treated with *m*-chloroperbenzoic acid. A product with similar chromatographic properties also was occasionally obtained when Dyfonate was incubated with liver microsomes in the presence of NADPH$_2$. On the basis of analytical, spectral, and chemical evidence, the structure of this oxygenated unknown was suggested to be a resonance hybrid or rapidly equilibrating tautomer mixture of the structures Ia and Ib shown below.



Ia Ib

In connection with our investigation of the stereochemistry of the oxidation of Dyfonate to its oxon we have determined that this oxygenated material is phenyl ethoxy(ethyl)phosphinyl disulfide (II) of structure given below. Proof for this conclusion is presented as follows.



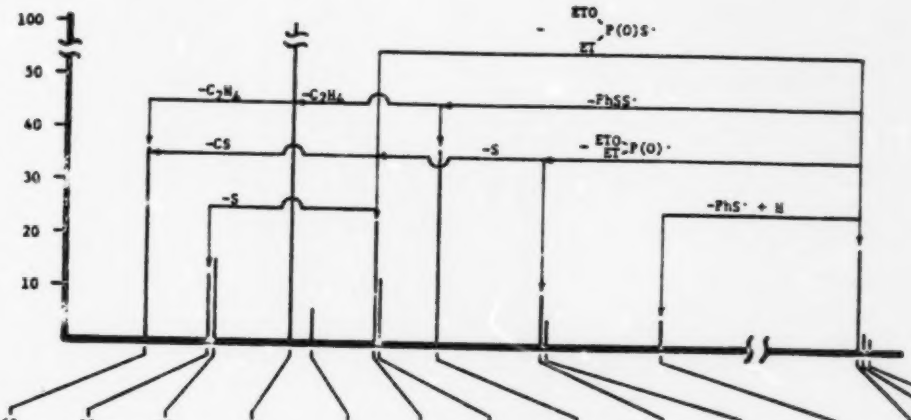
II

Structure Analysis - The oxygenated product (II) was obtained according to the procedure of McBain et al.[1] by treatment of Dyfonate with m-chloroperbenzoic acid and purification by column chromatograph. II also was prepared by reaction between benzenesulfenyl chloride and O-ethyl ethylphosphonothioic acid (ETP) following the general procedure of Schrader and Lorenz[2]. Comparison of the two samples of II obtained from the m-chloroperbenzoic acid oxidation of Dyfonate and the reaction of benzenesulfenyl chloride with ETP were identical. Proofs of identity were identical $R_f$ values in four tlc systems, identical $R_f$ values for the products (mainly ETP and diphenyl disulfide) formed by storage decomposition, and superimposibility of all spectra. The mass spectra provided the clearest evidence for the structure of compound II, and its identity with the m-chloroperbenzoic acid oxidation product of Dyfonate. Particularly significant is the presence of ions at m/e 141 ($PhS_2^+$) (Fig. 1). Comparison of these spectra with the spectra of benzenethiol and diphenyl disulfide explains part of the intensity of the ion at m/e 65 and the ions at m/e 77, 78, 109 and 110. Comparison of intensities also indicates that the ions at m/e 141 and 142 are not caused by the presence of a trace impurity of diphenyl disulfide. This latter compound decomposed initially by loss of both PhS- and $PhS_2$- in analogy to compound II.

The ir spectra of sulfides and disulfides are in general very similar, e.g. the spectra of diphenyl sulfide and diphenyl disulfide. An even closer analogy is the near identity of the ir spectra of aryl diethoxyphosphinothioyl disulfides and S-aryl O,O-diethyl phosphorodithioates. It is, therefore, reasonable that compound II and the Dyfonate oxon would have similar ir spectra.

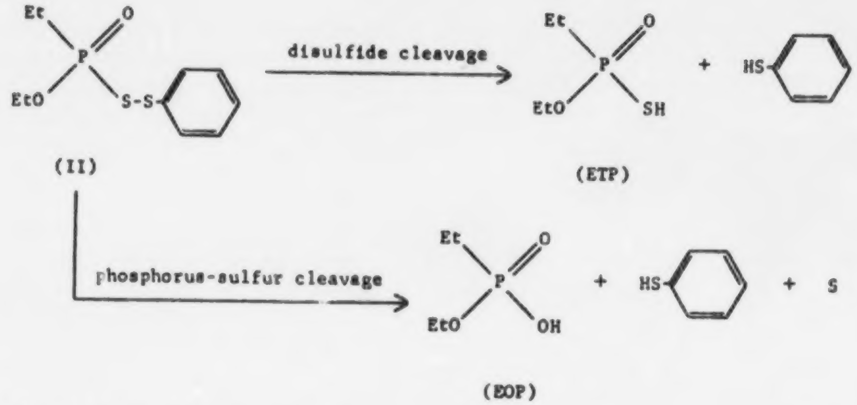
The $^{31}P$ nmr spectra of Dyfonate, the oxygenated Dyfonate and Dyfonate oxon show upfield shifts of 6.5, 20, and 58 ppm with respect to phosphorus oxide.[1] The $^{31}P$ chemical shifts are dependent on both the polarizability of the atoms attached to phosphorus and the double bond order. Shielding decreases as the polarizability of the atoms attached to phosphorus increases and as the percentage of the double bond character decreases. It is not clear why a combination of these factors should place the $^{31}P$ nmr shift of structures such as Ia, Ib, etc. intermediate between that of Dyfonate and Dyfonate oxon. However, on these arguments the $^{31}P$ chemical shift of compound II would be expected to be downfield from that of Dyfonate oxon.

The hydrolysis of the oxygenated Dyfonate (II) parallels the hydrolysis of alkyl and aryl dialkoxyphosphinyl disulfides because in these compounds the disulfide bond is cleaved by a variety of reagents. From this analogy, the observed decomposition of compound II predominantly to ETP by disulfide bond cleavage rather than to O-ethyl ethylphosphonic acid (EOP) by phosphorus-sulfur bond cleavage (below) is readily predictable. Bis-(dialkoxyphosphinyl) disulfides also cleave in a smilar manner. These compounds and bis-(dialkoxyphosphinothioyl) disulfides readily lose sulfur in the presence of a sulfur acceptor. Elemental sulfur was detected in the perbenzoic acid oxidation of Dyfonate.[1]



| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| M/e | | 65 | 77 | 78 | 93 | 97 | 109 | 110 | 121 | 141 | 142 | 154 | 262 | 263 | 264 |
| Fragment | | $PO_2H_2^{+\cdot}$<br>$C_5H_5^+$ | $C_6H_5^+$ | $C_6H_6^{+\cdot}$ | $C_2H_6PO_2^+$ | $PSO_2H_2^{+\cdot}$ | $\mathrm{PhS}^+$ | $\mathrm{PhSH}^{+\cdot}$ | $\mathrm{^{EtO}_{Et}{>}P{=}O^+}$ | $\mathrm{PhSS}^+$ | $\mathrm{PhSSH}^{+\cdot}$ | $\mathrm{^{EtO}_{Et}{>}POSH^{+\cdot}}$ | M | M+1 | M+2 |
| Rel. Int. % | Peracid Oxidation | 36.0 | 12.4 | 15.2 | 100 | 6.0 | 22.4 | 12.1 | 36.4 | 9.2 | 4.4 | 4.8 | 19.1 | 2.8 | 2.0 |
| | Cmpd II | 35.1 | 12.0 | 14.0 | 100 | 5.4 | 21.6 | 10.8 | 36.0 | 8.3 | 3.5 | 4.4 | 18.1 | 2.7 | 2.0 |

Fig. 1. Mass spectrum of compound II. Fragmentation pattern and relative intensities (average of all spectra) shown for the m-chloroperbenzoic acid oxidation product of Dyfonate and for compound II synthesized from benzenesulfenyl chloride and O-ethyl ethylphosphonothioic acid.

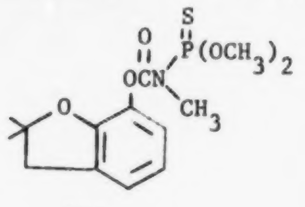


The failure to isolate Dyfonate oxon from the "oxygenated Dyfonate" is as expected for compound II but is not explained by structures such as Ia and Ib.

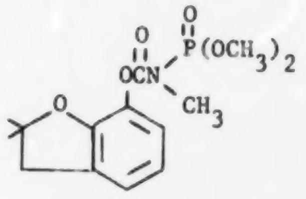
*Conclusion* - The proposed structure (II) for the *m*-chloroperbenzoic acid oxidation product is, therefore, consistent with all of the spectral and chemical evidence reported by McBain *et al.*[1] for their oxygenated Dyfonate. It appears likely that the oxygenated Dyfonate (II) is not a significant enzymatically-formed intermediate in microsomal systems. The $^{18}O$ labeling data from microsomal experiments of McBain *et al.*[3] on Dyfonate and similar data of Ptashne *et al.*[4] on parathion support this conclusion since the oxygen in *oxidatively formed* ETP and diethyl phosphorothioic acid comes from water. Caution must be thus exercised in drawing too close an analogy between peracid oxidation models and microsomal systems.

## 2. Oxidative Rearrangement of *N*-(Dimethoxyphosphinothioyl)carbamate Esters

*Introduction* - In connection with our studies on the comparative metabolism of 2,2-dimethyl-2,3-dihydrobenzofuranyl-7 *N*-methyl-*N*-(dimethoxyphosphinothioyl)carbamate (III) (see part B-3 ) in mammals and insects it became necessary to synthesize the corresponding *N*-dimethoxyphosphinyl derivative IV.

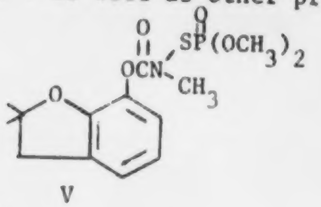


III

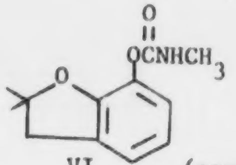


IV

Attempts to synthesize IV by the condensation reaction used for III failed and, therefore, it was decided to prepare IV by peracid oxidation of III. Treatment of III with m-chloroperbenzoic acid gave IV in about 40% yield but also gave an unexpected rearrangement product which subsequently was identified as the N-dimethoxyphosphinylthio derivative V. This section reports results leading to the identification of V as well as other products obtained from the oxidation of III.



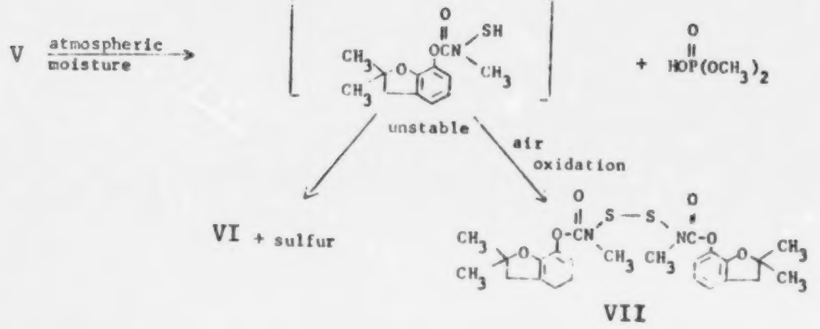
V



VI (carbofuran)

Structure Analysis - Treatment of III with m-chloroperbenzoic acid afforded IV and V as the principal products along with lesser amounts of VI (carbofuran) and starting material (III). Sulfur also was isolated. VI was identical to material synthesized independently from methyl isocyanate and the corresponding phenol. Attempts to synthesize IV by the method used for III failed and its structure was established from the following spectra data: IR, 1725 $cm^{-1}$ (C=O) and 1260 $cm^{-1}$ (P=O); NMR (δ, 60 MHz, $CDCl_3$) 3.28 (doublet, J = 8 Hz, 3H, $-NC\underline{H}_3$), and 6.8-7.2 (multiplet, 3H, aromatic protons); MS, m/e 329 ($M^+$, 8.8%), 166 (31.4%), 109 (100%), and 164 (10.8%). The structure of V was established from the following spectral data: IR, 1725 $cm^{-1}$ (C=O) and 1260 $cm^{-1}$ (P=O); NMR (δ, 60 MHz, $CDCl_3$), 3.45 (singlet, 3H, $-NC\underline{H}_3$), 3.95 (doublet, J = 13 Hz, 6H, $OC\underline{H}_3$) and relevant absorptions similar to IV; MS, m/e 361 ($M^+$, 10.6%), 198 (62.3%), 141 (100%) and 109 (34%). The downfield shift and the change to a singlet for $N-C\underline{H}_3$ absorption for V is consistent with the displacement of this moiety another atom away from phosphorus. The value of m/e 198 (62.3%) in the MS of V is consistent with $[O{=}\overset{+}{C}N(CH_3SP(O)(OCH_3)_2]$, m/e 166 (31.4%) for IV with $[O{=}\overset{+}{C}N(CH_3)P(O)(OCH_3)_2]$ and m/e 182 (29.4%) for III with $[O{=}\overset{+}{C}N(CH_3)P(S)(OCH_3)_2]$. Further fragmentation of each of these ions by the loss of methyl isocyanate produced: m/e 141 (100%) $[SP(O)(OCH_3)_2]^+$ for V which then lost sulfur to give m/e 109 (34%) $[OP(OCH_3)_2]^+$; m/e 109 (100%) $[OP(OCH_3)_2]^+$ for IV; and m/e 125 (100%) $[SP(OCH_3)_2]^+$ for III.

Further evidence for the structure of V was obtained from its decomposition on silica gel thin-layer plates. V evidently is unstable to prolonged atomspheric exposure on silica gel and decomposes mainly to a disulfide VII, the carbamate VI, and sulfur, presumably by the following scheme.



VII, mp 131-3°; NMR (δ, 60 MHz, $CDCl_3$), 3.45 (singlet, 6H, $-NC\underline{H}_3$), 3.05 (singlet, 4H, $-C\underline{H}_2$), 1.25 (singlet, 12H, $-C\underline{H}_3$), and 6.8-7.2 (mulciplet, 6H, aromatic protons); MS m/e 504 ($M^{+}$, 5.4%), 447 (2.1%), 390 (6.2%), 164 (27%), 163 (100%), 149 (10%), 135 (27%), and 107 (14.5%).

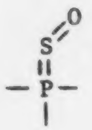
Oxidation of $^{14}C$-labeled II ($N^{14}CH_3$) with m-chloroperbenzoic acid also was carried out for quantitative determination of the products by TLC. The results are presented in the table below.

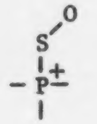
Table 1. YIELDS OF PRODUCTS FROM THE OXIDATION OF III.

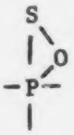
| Oxidation products of III[a] | | | Decomposition products of V[b] | | |
|---|---|---|---|---|---|
| Compound | $R_f$ | % Radioactivity | Compound | $R_f$ | % Radioactivity |
| III | 0.56 | 11.5 | VI | 0.34 | 31.4 |
| VI | 0.34 | 13.7 | IV[c] | 0.08 | 6.4 |
| V | 0.15 | 35.0 | VII | 0.60 | 62.2 |
| IV | 0.08 | 39.8 | | | |

[a] Based on 92% total recovery.
[b] Based on 97% total recovery.
[c] It is uncertain whether IV is formed from V or whether this small amount was present as a contaminant in V prior to TLC exposure.

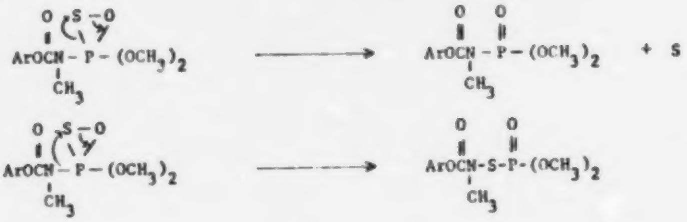
Conclusion - The above information clearly reveals that oxidation of III produces a rearrangement product (V) similar to that obtained from the oxidation of Dyfonate. The results may be explained on the basis that oxidation of III proceeds through an unstable S-oxide intermediate[5] which may be written in the following resonance and tautomeric forms.







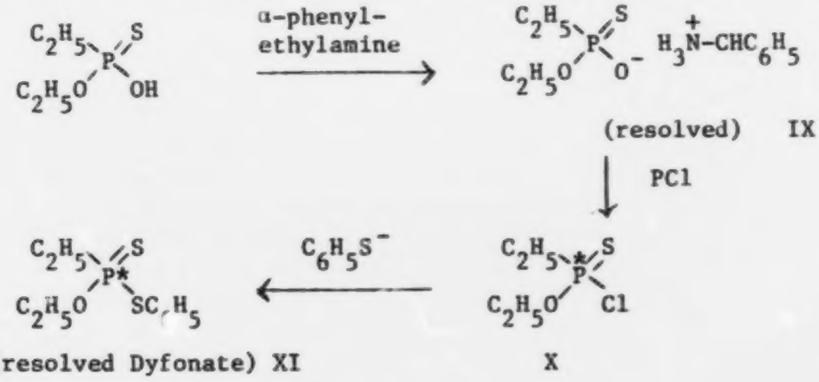
This intermediate explains the formation of IV and V as follows.



### 3. The Resolution and Determination of the Absolute Configuration of Dyfonate and Related Compounds

Introduction - In order to study the stereochemistry of the PS to PO activation reaction of thionate esters it became necessary to resolve the isomers of Dyfonate and determine their absolute configurations. The results from this investigation are presented as follows.

Synthesis and Resolution - The starting material in the synthesis of resolved Dyfonate was O-ethyl ethylphosphonic acid as shown below.


α-phenyl-
ethylamine
(resolved) IX
PCl
$C_6H_5S^-$
(resolved Dyfonate) XI
X

Resolution of the (+)- and (-)- isomers of VIII was achieved by fractional cyrstallization of its α-phenylethylamine salt IX. The free resolved acid generated from IX was treated with phosphorus pentachloride to afford the chloride X which was subsequently reacted with benzenethiolate to give resolved Dyfonate (XI). The last two reactions took place with minimum racemization. Synthesis of resolved Dyfonate oxon (XII) was obtained by reaction of resolved VIII with phenyldiazonium chloride.

Determination of Absolute Configuration - The (-)-rotating isomer of IX was subjected to X-ray analysis. The crystal used for X-ray analysis had dimensions 0.10 X 0.45 X 0.10 mm. Precession and Weissenberg photographs showed mono-clinic symmetry and systematic extinctions 0k0 with k = 2n + 1. Since the molecule is chiral, the space group is uniquely determined as $P2_1$.

The cell constants, $\underline{a}$ = 11.500 (13), $\underline{b}$ = 6.691(7), $\underline{c}$ = 11.327(13) A, and β = 115.08(3)°, were determined from a least-squares fit of 12 carefully centered relections measured on a Picker automatic diffractometer (MoK $\bar{\alpha}$, λ = 0.71069 A). The density, 1.15 g $cm^{-3}$, measured by flotation in hexane/carbon tetrachloride, agrees with the value 1.158 g $cm^{-3}$ calculated for two molecules of $C_{12}H_{22}NO_2PS$ in the unit cell.

Three-dimensional intensity data were collected on the above diffractometer, MoK $\bar{\alpha}$ radiation mode monochromatic by Bragg reflection from a graphite crystal, scanning reflections in the 2θ-θ mode at a scan rate of 1°/min and a scan range of 2.0°, and taking background counts of 10 sec at each end of the scan. Of 688 unique reflections having 2θ values less than 37°, 632 remained after rejection of those with intensities less than 1.5σ. These were put on a common scale using the intensities of three standards (collected every 75 reflections) and corrected for Lorentz-polarization effects.

The structure was solved by the heavy-atom method, successive cycles of Fourier synthesis, and subsequently full-matrix least-squares refinement. Sulfur and phosphorus atoms were refined anisotropically, whereas other atoms were refined isotropically. The final residue R was 10.5%. All hydrogen atoms were observed on the difference Fourier map, but not included in the refinement.

The absolute configuration of the (-)-$\underline{O}$-ethyl ethylphosphonothioate moiety was established as $\underline{S}$ by relation to the known configuration of the $\underline{S}$-(-)-α-phenylethylammonium ion. A stereoscopic view of the molecule (both cation and anion) is shown in Fig. 2. The quaternary ammonium protons form hydrogen bonds to three neighboring anions, one to the sulfur (3.29 Å) and two to the charge-bearing oxygens (2.78 and 2.77 Å) of two separate anions.

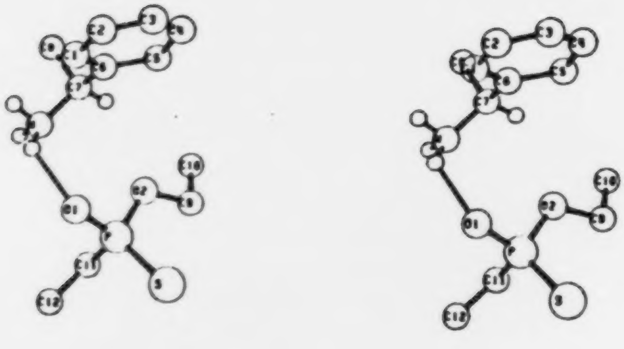


Fig. 2. Stereoscopic view of $\alpha$-phenylethylammonium salt of O-ethyl ethylphosphonothioic acid. The double line indicates the hydrogen bond.

Because Dyfonate is a liquid at room temperature, it was necessary to obtain an analog which was a crystalline solid for X-ray analysis.

Fortunately, O-ethyl S-p-bromophenyl ethylphosphonodithioate (XIII) also turned out to be a solid, albeit of rather low melting point (31.5°C). Colorless crystals of the (-)-enantiomer of XIII were obtained by slow evaporation of a water-methanol solution, and subjected to X-ray analysis. Preliminary X-ray examination of the crystals of XIII (performed at 19°C) revealed a triclinic symmetry and the space group was determined as P1. Assumption of two molecules of XIII per unit cell was in complete agreement with the density both calculated from X-ray data and that obtained by the flotation method (in $CCl_4$-$HCCl_3$).

Although the two independent molecules in the unit cell have the same chirality, they are apparently related by a pseudo center-of-symmetry (excluding the ethyl and ethoxyl moieties in XIII). If the two molecules were perfectly related by the pseudo center-of-symmetry in the crystal, it would be impossible to determine the absolute configuration. Fortunately the nature of the molecular arrangement in the crystal is such that the overall geometry makes the heavy-atoms in the two molecules in the unit cell slightly off from being centrosymmetrical and this phenomenon made the determination of the absolute configuration of XIII possible.

The structures were solved by the heavy-atom method, through successive cycles of full-matrix least-squares calculations. Bromine, sulfur and phosphorus atoms were refined anisotropically whereas others were refined isotropically. The absolute configuration of the chiral phosphorus center of the (-)-rotomer of XIII determined via the anomalous dispersion effects of the bromine, sulfur and phosphorus was eventurally assigned as S. A stereoscopic view of one of the two molecules in the unit cell is shown in Figure 3. The other molecule is not included in the figure because the two molecules have identical configuration and chirality.

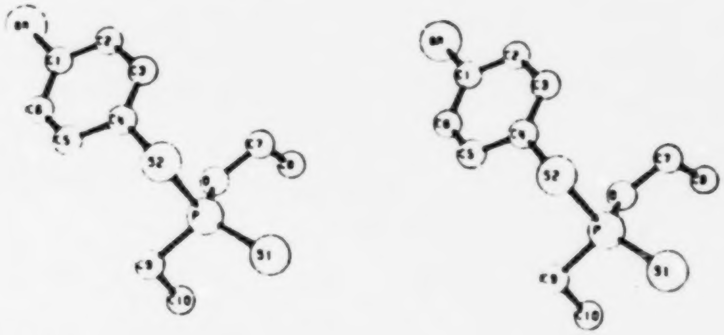


Fig. 3. Stereoscopic view of one of the two molecules S(-)-O-ethyl S-p-bromophenyl ethylphosphonodithioate ester (XIII) in the unit cell.

The absolute configuration of the isomers of Dyfonate oxon (XII) was established by nucleophilic substitution reaction of the sodium salt of (-)-O-ethyl ethylphosphonothioic acid (VIII) with phenyldiazonium chloride. Because bonds attached to the phosphorus atom in the resolved acid are not affected by this reaction, the absolute configuration of XII is identical to that of VIII.

Conclusion - The absolute configuration of the chiral isomers of O-ethyl ethylphosphonothioic acid and O-ethyl p-bromophenyl ethylphosphonodithioate has been established by X-ray crystallography. Since the stereochemistry of the reactions leading to Dyfonate and the p-bromo analog are the same, the absolute configuration of Dyfonate also was established. The absolute configuration of chiral Dyfonate oxon was determined from its relationship to chiral O-ethyl ethylphosphonothioic acid.

The assignment of absolute configuration of the individual chiral isomers of these compounds now makes it possible to study the mechanism of the PS to PO activation reaction in biological and model oxidation systems. This work is currently in progress.

## 4. References

1. McBain, J. B., J. Yamamoto, and J. E. Casida. Oxygenated Intermediate in Peracid and Microsomal Oxidations of the Organophosphonothionate Insecticide Dyfonate®. Life Sciences 10: 1311-9, 1971.

2. Schrader, G., and W. Lorenz. Ger. Pat. 1,103,324 (October 20, 1959). Sulfenylthiophosphate and -thiophosphonate esters. Chem. Abs. 56:5838c, 1962.

3. McBain, J. B., I. Yamamoto, J. E. Casida. Mechanism of Activation And Deactivation of Dyfonate® (O-Ethyl S-phenyl Ethyl phosphonodithioate) by Rat Liver Microsomes. Life Sciences 10:947-54, 1971.

4. Ptoshne, K. A., R. H. Wolcott, and R. A. Neal. Oxygen-18 Studies on the Chemical Mechanisms of the Mixed Function Oxidase Catalyzed Desulfuration and Dearylation Reactions of Parathion. J. Pharmocol. Exp. Therap. 179:380-5, 1971.

5. Herriott, A. W. Peroxy Acid Oxidation of Phosphinothioates, a Reversal of Stereochemistry. J. Amer. Chem. Soc. 93:3304-5, 1971.

## G. STUDIES OF INSECTICIDE SYNERGISM AND INSECT GROWTH REGULATORS

During the course of our studies on insecticide synergists we discovered that some of the intermediates which were synthesized on route to substituted 1,3-benzodioxoles were not only effective synergists but also were effective in preventing the normal development of mosquito larvae into adults. For this reason, the results obtained on insecticide synergism and insect growth regulators are combined.

### 1. Carbaryl Synergism by Substituted 1,3-Benzodioxoles and Related Compounds and Inhibition of Rat liver Mixed Function Oxidase

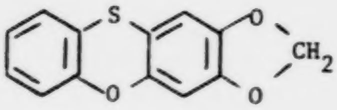
Introduction - Several years ago Hansch[1], by means of multiple regression analysis of data on the synergism of the toxicity of carbaryl to houseflies by a series of ring-substituted 1,3-benzodioxole derivatives, showed that synergistic activity may be correlated with the parameters $\pi$ and $\sigma\cdot$ for the substituents according to the equation below.

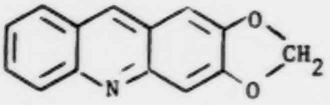
$$\log SR_5 = -0.195\,\pi^2 + 0.670\,\pi + 1.316\,\sigma\cdot + 1.612$$

$$\underline{n} = 13,\ \underline{r} = 0.929,\ \underline{s} = 0.171$$

In this equation, $SR_5$ is the degree of synergism obtained using 5 parts of synergist to 1 part carbaryl, $\pi$ is essentially a "hydrophobic binding" constant, $\sigma\cdot$ is a parameter similar to Hammett's $\sigma$ constant obtained from the radical phenylation of substituted benzene derivative, $\underline{n}$ is the number of compounds, $\underline{r}$ is the correlation coefficient, and $\underline{s}$ is the standard deviation from the regression.

From this regression equation, several 1,3-benzodioxole derivatives, including those given below, were predicted as potential synergists.





This section is concerned with the synthesis of compounds of this type, and evaluation of their synergistic activity and inhibition of rat liver microsomal oxidase.

Toxicological Data - All compounds indicated in Tables 1-3 were synthesized by literature methods or purchased from commercial sources. Structures of all compounds were confirmed by pmr and usually mass spectroscopy.

The synergists were evaluated by the topical application of a 5:1 (w/w) combination of synergist and carbaryl (1-naphthyl *N*-methylcarbamate) in acetone to the thoraces of 3-day-old female NAIDM houseflies, *Musca domestica*. All synergists were nontoxic at maximum concentrations employed in this work. Log $SR_5$ data in the tables are based on an $LD_{50}$ value for unsynergized carbaryl of 50 μg/female fly. Aldrin epoxidation[2] was used as the index of microsomal oxidase activity, and female rat liver was the enzyme source. Inhibitor potencies of the various compounds were determined by measuring per cent inhibition of epoxidation at $5 \times 10^{-4}$ *M*. $I_{50}$ values were determined by using 3 or 4 different concentrations.

Data for the synergism of carbaryl and the in vitro inhibition of female rat liver microsomal oxidase activity by compounds closely related to those specifically suggested as potentially effective synergists by Hansch[1] are given in Table 1. These compounds are methylenedioxy derivatives of 6-chloro-acridine (**1** and **2**), anthraquinone (**3**), anthracene (**4**), and phenazine (**5**). Four of the five compounds in Table 1 have high $SR_5$ values, particularly when their relatively high molecular weights are taken into account. Semiquantitatively, the data support the prediction by Hansch that methylenedioxyaryls of the type represented by **1**-**5** should be effective carbamate synergists.

The ease with which acridines, phenazines, anthracenes, and anthraquinones form radical species is well known and, therefore, compounds **1**-**5** can be expected to have high $\sigma\cdot$ values. In addition, since phenothiazine, dibenzofuran, and methylacridine have high log P values[1], i.e., in the vicinity of 4±0.1, and in light of their close structural relationships it is not unreasonable to assume similar high log P values for compounds **1**-**5**. Thus, based on equation 1, compounds **1**-**5** are expected to have high synergistic activity.

On route to the synthesis of compounds **1**, **2**, and **5** and the attempted synthesis of some related phenothiazine analogs, a number of 4,5-disubstituted 1,3-benzodioxoles whose structures are indicated in Table 2 were prepared as intermediates. Since compounds of this general type were used in the structure-activity analysis by Hansch[1], the intermediates listed in Table 2 were examined for synergistic activity. Several of the compounds, particularly **11**, **12**, and **14**, proved to be exceptionally effective in synergizing the toxicity of carbaryl to houseflies. Unfortunately, data were not available to calculate $\sigma\cdot$ for the various X and Y substitutents (exception of $NO_2$) and it was not possible to compare calculated (from equation 1) log $SR_5$ values with experimental values. Known chemistry suggests high $\sigma\cdot$ values for most of these substituents, particularly the *O*-phenyl and *S*-phenyl moieties where additional resonance structure for the substituted 1,3-benzodioxole radical may be envisioned. $\pi$ values for *O*-phenyl and *S*-phenyl substituents also are not known but they undoubtedly are favorable for synergism. Moderately large changes in synergistic activity accompanying small changes in

Table 1. TOXICOLOGICAL AND OXIDASE INHIBITION DATA FOR FUSED POLYCYCLIC 1,3-BENZODIOXOLES

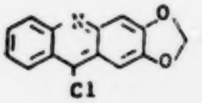
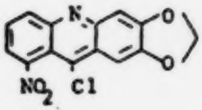
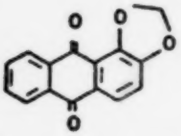
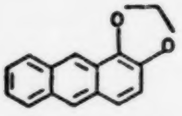
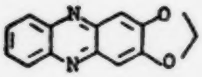
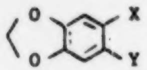
| No. | Compound | MP °C | $LD_{50}$ mg/kg carbaryl 5:1 | Log $SR_5$ | % Microsomal oxidase inhibition at 5 X $10^{-4}$ M |
|---|---|---|---|---|---|
| 1 |  $\cdot H_2O$ | 178-80 | 34 | 1.87 | 13 ± 11 |
| 2 |  | >300 | >48 | - | 3 ± 3 |
| 3 |  | 258-60 | 10 | 2.4 | 22.25 ± 2.5 |
| 4 |  | 130.5-132 | 9.4 | 2.43 | 27 ± 3 |
| 5 |  | 220 | 32.1 | 1.89 | 25 ± 7 |

Table 2. TOXICOLOGICAL AND OXIDASE INHIBITION DATA FOR 3,4-DISUBSTITUTED 1,3-DIOXOLES



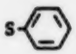
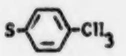
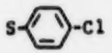
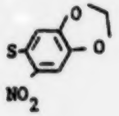
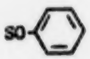
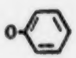
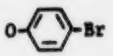
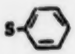
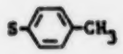
| No. | X | Y | MP °C | $LD_{50}$ carbaryl (mg/kg 5:1) | Log $SR_5$ | % Oxidase inhibition at $5 \times 10^{-4}$ M |
|---|---|---|---|---|---|---|
| 6 |  | $NO_2$ | 137.5-38 | 18.1 | 2.14 | 15 ± 9 |
| 7 |  | $NO_2$ | 181-82 | 70.1 | 1.55 | 30 ± 10 |
| 8 |  | $NO_2$ | 161.5-62.5 | 54.2 | 1.67 | 22 ± 10 |
| 9 |  | $NO_2$ | 259-61 | 25.1 | 2.00 | -5 ± 9 |
| 10 |  | $NO_2$ | 125-26 | 70.0 | 1.55 | 49.5 |
| 11 | SEt | $NO_2$ | 125-26 | 5.2 | 2.68 | 95.5 ± 0.5 |
| 12 |  | $NO_2$ | 140.5-41.5 | 5.04 | 2.70 | 5 ± 1.5 |
| 13 |  | $NO_2$ | 134-35 | 12.9 | 2.29 | 71.0 ± 2 |
| 14 |  | NHAc | 103.5-104.5 | 4.90 | 2.71 | 53 ± 7 |
| 15 |  | NHAc | 161-2 | 30.9 | 1.91 | 84 ± 4 |

Table 2 continued

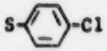
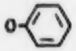
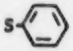
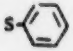
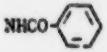
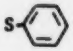
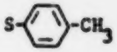
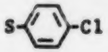
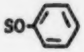
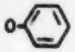
| | | | | | | |
|---|---|---|---|---|---|---|
| 16 |  | NHAc | 137.5-38.5 | 21.1 | 2.07 | 64.6 ± 7 |
| 17 |  | NHAc | 128.5-29.5 | 33.1 | 1.88 | 7 ± 2 |
| 18 |  | NHCOEt | 70.5-71.5 | 11.5 | 2.34 | 78.2 ± 4.8 |
| 19 |  |  | 132-133.5 | 40.1 | 1.79 | 80 ± 1 |
| 20 |  | $NH_2 \cdot HCl$ | 158-61 | | | 83.5 ± 1.3 |
| 21 |  | $NH_2 \cdot HCl$ | 156-59 | | | 90 ± 1.2 |
| 22 |  | $NH_2 \cdot HCl$ | 163-65 | | | 76.5 ± 6 |
| 23 |  | $NH_2 \cdot HCl$ | 126-29 | | | 65 ± 5.5 |
| 24 |  | $NH_2 \cdot HCl$ | 146-50 | | | - |

Table 3. OXIDASE INHIBITION DATA FOR SUBSTITUTED 1,3-BENZODIOXOLES AND THEIR CORRESPONDING CATECHOLS

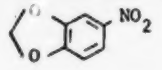
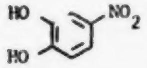
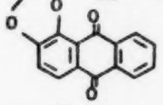
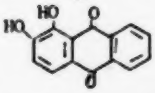
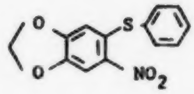
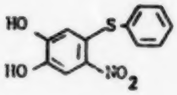
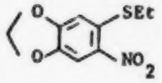
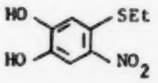
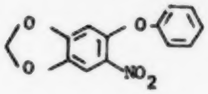
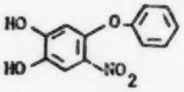
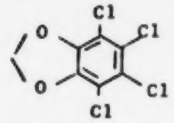
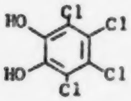
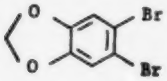
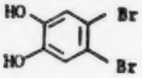
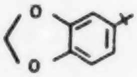
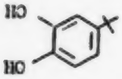
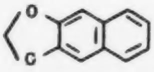
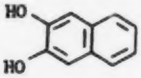
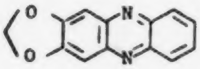
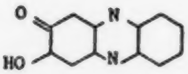
| No. | Compound | % Inhibition at 5 X $10^{-4}$ M | $I_{50}$ | Log $SR_5$ |
|---|---|---|---|---|
| 25 |  | -6 ± 6 | >2 X $10^{-3}$ | 2.40 |
| 26 |  | 31 ± 6 | >2 X $10^{-3}$ | |
| 3 |  | 22.25 ± 2.5 | 1.18±0.38 X $10^{-3}$ | 2.4 |
| 27 |  | 13 ± 3 | 1.31±0.31 X $10^{-3}$ | |
| 6 |  | 15 ± 9 | 3.6±0 X $10^{-4}$ | 2.14 |
| 28 |  | 91.5 ± 1 | 1.08±0.1 X $10^{-4}$ | |
| 11 |  | 45.5 ± 0.5 | 3.9±0.9 X $10^{-5}$ | 2.68 |
| 29 |  | 48 ± 5 | 7.4±1.4 X $10^{-4}$ | |
| 12 |  | 5 ± 1.5 | >2 X $10^{-3}$ | 2.70 |
| 30 |  | 88.9 ± 11.5 | 3.63±0.5 X $10^{-4}$ | |

Table 3 continued

| | | | | |
|---|---|---|---|---|
| 31 |  | 3 ± 2 | >2 X $10^{-3}$ | 2.36 |
| 32 |  | 54.0 ± 9 | 4.15±1.1 X $10^{-4}$ | |
| 33 |  | 49 ± 0 | 4.99 X $10^{-4}$ | 2.44 |
| 34 |  | 94.8 ± 1.2 | 3.7±0.6 X $10^{-5}$ | |
| 35 |  | 44.5 ± 4.75 | 5.0±0.3 X $10^{-4}$ | 1.88 |
| 36 |  | 71.6 ± 2.0 | 2.8±0 X $10^{-4}$ | |
| 37 |  | 49.9 ± 1.9 | 6.75±1.75 X $10^{-4}$ | 2.64 |
| 38 |  | 61.25 ± 1.75 | 3.25±0.1 X $10^{-4}$ | |
| 5 |  | 25 ± 7 | 8.75±1.25 X $10^{-4}$ | 1.89 |
| 39 |  | 52.5 ± 4 | 3.0±0.4 X $10^{-4}$ | |

structure within each sub-series, e.g., compare 14 and 15, 6 and 7, etc., however, are difficult to explain.

Inhibition of Microsomal Oxidase - Available evidence indicates that the 1,3-benzodioxoles prevent oxidative metabolism by acting as alternative substrates and/or binding with the microsomal enzyme system. Examination of the data in Tables 1 and 2 shows that there is little relationship, if any, between in vitro inhibition of rat liver microsomal aldrin epoxidation and in vivo synergistic activity. For example, compound 12, one of the most potent synergists of the series (log $SR_5$ 2.70), produced very little inhibition of aldrin epoxidation at the concentration of 5 X $10^{-4}$M. Similarly, 9, a moderately good synergist, was virtually inactive as an inhibitor at the same concentration and in some of the replicated measurements appeared to cause enhancement of the rate of aldrin epoxidation by the enzyme. The most effective inhibitor among the compounds in Tables 1 and 2 was 11, a material which also happened to be a very effective synergist.

Although a number of factors above may contribute to the discrepancy between synergistic activity and inhibition, the possibility remains that the 1,3-benzodioxole derivatives alone are not totally responsible for synergism. Microsomal oxidases are known to catalyze the demethylenation of the methylenedioxy moiety in 1,3-benzodioxoles to yield formic acid and catechols[3,4] and the suggestion has been made that the catechols also may be involved in preventing detoxication of an insecticide[4]. Experimental evidence for this possibility, however, has not been provided.

Therefore, a series of methylenedioxyphenyl compounds and their corresponding catechols were examined as inhibitors of microsomal aldrin epoxidation in an attempt to define the nature of the structural features upon which inhibitory action depends. In Table 3 are listed the values for the per cent inhibition at a concentration of 5 X $10^{-4}$M test compound and the molar $I_{50}$ (molar concentration to inhibit 50% of a fixed amount of oxidase) for each of the substances examined. For the pairs represented by 25 and 26, 3 and 27, 35 and 36, 37 and 38, the methylenedioxyphenyl compound and corresponding catechol were each approximately equal in their effectiveness in inhibiting aldrin epoxidation. The methylenedioxyphenyl compounds 6 and 11 were more potent than their respective catechols, 28 and 29, but in four cases (5 and 39, 12 and 30, 31 and 32, 33 and 34) the catechol was the more potent inhibitor. The most effective inhibitors tested were 4-nitro-5-ethylthio-1,3-benzodioxole (11) and 4,5-dibromocatechol (34) with $I_{50}$ values of 3.9 X $10^{-5}$M and 3.7 X $10^{-5}$M, respectively. The compounds varied considerably in aqueous solubility which made interpretation of small differences in inhibition values of questionable validity. However, this does not obscure the interesting finding that certain of the cataechols are as potent, or even more potent, as inhibitors of aldrin epoxidation than the corresponding methylenedioxyphenyl compound. It should be pointed out that none of the catechols were active in synergizing the toxicity of carbaryl to houseflies. This, of course, is not surprising since these materials are expected to be transient in biological systems.

Conclusion - The synergism data in Table 1 for several of the polyaromatic 1,3-dioxole derivatives confirmed predictions made earlier by Hansch. Several of the 4,5-disubstituted-1,3-benzodioxoles were outstanding in their effectiveness in synergizing the toxicity of carbaryl. A direct relationship between synergistic activity and inhibition of microsomal aldrin epoxidation was not observed. However, data on inhibition of microsomal oxidase revealed that the corresponding catechols are often more potent inhibitors than the related 1,3-benzodioxoles and it is possible that in some cases the catechol may contribute to synergistic activity.

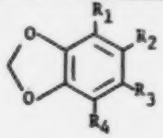
## 2. The Effect of 1,3-Benzodioxoles, Catechols, and Quinones in Mosquito Larvae

Introduction - Apparent juvenile hormone (JH) effects have been demonstrated in houseflies[5] and other insects[6,7] after treatment with 1,3-benzodioxole synergists. These synergists are known to inhibit insect mixed function oxidase systems, insect tyrosinase systems, and oxidative phosphorylation. Various oxidative processes involved in insect metamorphoses show differing activities at different stages of development[8,9]. Interference with any of these could well result in apparent JH activity. Further, as inhibitors of oxidative processes, materials such as MON-0585, 2,6-di-*t*-butyl-4-($\alpha,\alpha$-dimethylbenzyl) phenol, and other radical inhibitors may affect the titre of natural JH by interfering with the degradation of the hormone even though the principal metabolites of externally applied JH are hydrolytic in nature[10]. Because of the apparent JH activity of MON-0585, it was decided to examine various substituted 1,3-benzodioxoles discussed in the preceding section. This report is concerned with the effect on mosquito larvae of three classes of inhibitors of oxidative processes, i.e., substituted 1,3-benzodioxoles, catechols, and quinones.

Toxicological Data - All compounds were synthesized according to literature methods or purchased from commercial sources. Several of the compounds were available from the project described in the preceding section. Structures were confirmed by pmr and usually by mass spectroscopy. The method of mosquito larvae (*Culex pipiens quinquefasciatus*) bioassay was essentially that of Sacher[11] for continuous exposure except that a larger volume of water was used for reasons discussed later.

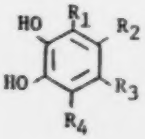
Results were tabulated for % 24-hour larval mortality, % total larval mortality, % non-emerging adults and % emerging adults. Larvae that had not progressed to the pupal stage after 21 days were classified as dead.

Table 4. TOXICOLOGICAL DATA FOR SUBSTITUTED 1,3-BENZODIOXOLES AGAINST 3RD-INSTAR CULEX PIPIENS LARVAE.



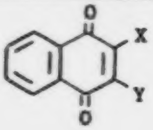
| No. | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Conc. ppm | 24 Hr kill | % Dead larvae | % Dead pupae | % Dead adults | % Live adults |
|---|---|---|---|---|---|---|---|---|---|---|
| 40 | H | $NO_2$ | H | H | 1 | 0 | 25 | 5 | 15 | 55 |
| 41 | Cl | Cl | Cl | Cl | 1 | 0 | 100 | 0 | 0 | 0 |
| | | | | | 0.1 | 0 | 25 | 0 | 0 | 75 |
| 42 | H | t-Bu | H | H | .1 | 0 | 30 | 0 | 0 | 70 |
| 43 | H | $NO_2$ | SET | H | 1 | 100 | 100 | 0 | 0 | 0 |
| | | | | | .1 | 0 | 90 | 0 | 0 | 10 |
| 44 | H | $SC_6H_5$ | $NO_2$ | H | 1 | 100 | 100 | 0 | 0 | 0 |
| | | | | | .1 | 0 | 5 | 5 | 5 | 85 |
| 45 | H | $OC_6H_5$ | $NO_2$ | H | 1 | 5 | 75 | 5 | 20 | 0 |
| | | | | | .1 | 0 | 85 | 0 | 0 | 15 |
| 46 | H | Br | $NO_2$ | H | 1 | 0 | 40 | 0 | 0 | 60 |
| 47 | H | $OC_6H_4$p-Br | $NO_2$ | H | 1 | 25 | 95 | 0 | 0 | 5 |
| | | | | | .1 | 40 | 50 | 5 | 0 | 45 |
| 48 | H | $SC_6H_4$p-Mc | $NO_2$ | H | 1 | 40 | 50 | 25 | 0 | 25 |
| 49 | H | Bf | Bf | H | 1 | 100 | 100 | 0 | 0 | 0 |
| | | | | | .1 | 0 | 5 | 0 | 5 | 90 |
| 50 | H | $S(O)C_6H_5$ | $NO_2$ | H | 1 | 0 | 15 | 0 | 5 | 80 |

Table 5. TOXICOLOGICAL DATA FOR SUBSTITUTED BENZCATECHOLS AGAINST 3RD-INSTAR CULEX PIPIENS LARVAE.



| No. | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Conc. ppm | 24 Hr kill | % Dead larvae | % Dead pupae | % Dead adults | % Live adults |
|---|---|---|---|---|---|---|---|---|---|---|
| 51 | H | $NO_2$ | SET | H | 1 | 100 | 100 | 0 | 0 | 0 |
| | | | | | 0.1 | 0 | 15 | 0 | 5 | 80 |
| 52 | Cl | Cl | Cl | Cl | 1 | 100 | 100 | 0 | 0 | 0 |
| | | | | | .1 | 0 | 10 | 0 | 10 | 80 |
| 53 | H | $OC_6H_5$ | $NO_2$ | H | 1 | 100 | 100 | 0 | 0 | 0 |
| | | | | | .1 | 100 | 100 | 0 | 0 | 0 |
| | | | | | .05 | 100 | 100 | 0 | 0 | 0 |
| | | | | | .02 | 50 | 60 | 0 | 0 | 40 |
| 54 | H | H | H | H | 1 | 35 | 80 | 0 | 5 | 15 |
| 55 | H | $NO_2$ | H | H | 1 | 0 | 45 | 5 | 5 | 45 |
| 56 | H | $NO_2$ | Br | H | 1 | 10 | 15 | 0 | 0 | 85 |
| 57 | H | Br | Br | H | .1 | 0 | 30 | 0 | 5 | 75 |
| 58 | H | _t_-Bu | H | H | 1 | 0 | 85 | 0 | 10 | 5 |
| | | | | | .1 | 0 | 25 | 0 | 5 | 70 |
| 59 | Br | H | _t_-Bu | H | .1 | 0 | 15 | 0 | 5 | 80 |
| 60 | OH | H | H | H | 1 | 0 | 85 | 5 | 0 | 10 |

Table 6. TOXICOLOGICAL DATA FOR SUBSTITUTED 1,4-NAPHTHAQUINONES AND 2,3-NAPHTHALENEDIOLS AGAINST 3RD-INSTAR CULEX PIPIENS LARVAE.



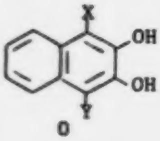
| No. | X | Y | Conc. ppm | 24 Hr kill | % Dead larvae | % Dead pupae | % Dead adults | % Live adults |
|---|---|---|---|---|---|---|---|---|
| 61 | Cl | Cl | 0.1 | 60 | 100 | 0 | 0 | 0 |
| | | | .05 | 10 | 85 | 5 | 0 | 10 |
| 62 | $CH_3$ | H | 1 | 45 | 75 | 0 | 0 | 75 |
| 63 | OH | H | 1 | 0 | 35 | 0 | 0 | 65 |
| 64 | $SCH_3$ | H | 1 | 100 | 100 | 0 | 0 | 0 |
| | | | .1 | 0 | 0 | 0 | 0 | 100 |
| 65 | $OCH_3$ | $OCH_3$ | 1 | 0 | 0 | 0 | 0 | 100 |
| 66 | $SC_6H_5$ | $SC_6H_5$ | 1 | 0 | 10 | 0 | 40 | 50 |
| | | | |  | | | | |
| 67 | H | H | .1 | 0 | 5 | 0 | 5 | 90 |
| 68 | Cl | Cl | .1 | 5 | 30 | 0 | 0 | 70 |
| 69 | Br | Br | .1 | 0 | 55 | 45 | 0 | 0 |
| | | | .02 | 0 | 0 | 15 | 5 | 80 |

Table 7. TOXICOLOGICAL DATA FOR MISCELLANEOUS RADICAL INHIBITORS AGAINST 3RD-INSTAR CULEX PIPIENS LARVAE

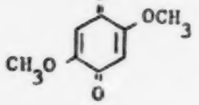
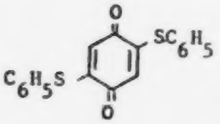
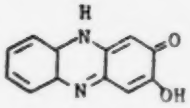
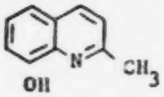
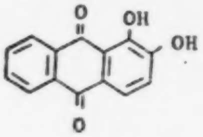
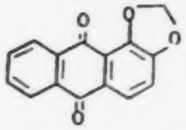
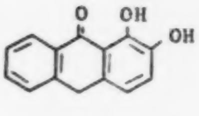
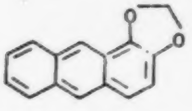
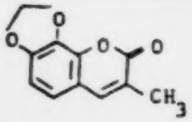
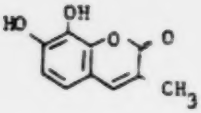
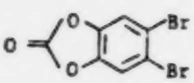
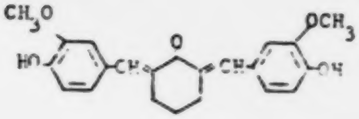
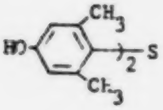
| No. | Compound | Conc. ppm | 24 Hr kill | % Dead larvae | % Dead pupae | % Dead adults | % Live adults |
|---|---|---|---|---|---|---|---|
| 70 |  | 1 | 5 | 95 | 0 | 0 | 5 |
| 71 |  | 1 | 0 | 0 | 0 | 0 | 100 |
| 72 |  | 1 | 0 | 75 | 5 | 0 | 20 |
| 73 |  | 1 | 0 | 85 | 10 | 0 | 5 |
|  |  | 0.5 | 5 | 35 | 5 | 5 | 50 |
| 74 |  | .1 | 100 | 0 | 0 | 0 | 0 |
|  |  | .02 | 0 | 20 | 10 | 0 | 70 |
| 75 |  | 1 | 100 | 0 | 0 | 0 | 0 |
|  |  | .1 | 5 | 20 | 5 | 0 | 75 |
| 76 |  | .1 | 100 | 0 | 0 | 0 | 0 |
|  |  | .02 | 0 | 45 | 5 | 0 | 50 |
| 77 |  | 1 | 100 | 0 | 0 | 0 | 0 |
|  |  | .1 | 10 | 0 | 0 | 5 | 85 |

Table 7 continued.

| No. | Compound | Conc. ppm | 24 Hr kill | % Dead larvae | % Dead pupae | % Dead adults | % Live adults |
|---|---|---|---|---|---|---|---|
| 78 |  | 1 | 100 | 0 | 0 | 0 | 0 |
| | | .1 | 0 | 10 | 5 | 0 | 85 |
| 79 |  | 1 | 0 | 15 | 0 | 0 | 85 |
| 80 |  | .1 | 100 | 0 | 0 | 0 | 0 |
| | | .05 | 5 | 30 | 10 | 5 | 55 |
| | | .02 | 0 | 0 | 0 | 0 | 100 |
| 81 |  | .1 | 0 | 35 | 50 | 15 | 0 |
| | | .02 | 0 | 15 | 5 | 5 | 75 |
| 82 |  | .1 | 5 | 30 | 40 | 0 | 5 |
| | | .05 | 0 | 10 | 5 | 0 | 85 |
| 83 | MON-0585 | .1 | 0 | 0 | 50 | 0 | 50 |

In other experiments the volume of the aqueous test solution was varied, but the concentration of the compound was kept constant. Conditions employed were 20 larvae in 40 ml of water in a 50 ml glass beaker and 20 larvae in 200 ml of water in a 400 ml glass beaker. In some cases 4th-instar larvae also were used.

All classes of compounds investigated showed larvicidal activity although the type of activity varied. Mortality data against the later 3rd-instar of *Culex pipiens* are recorded in Tables 4-7. For these larvae ecdysis occurred for over 50% of the control larvae within 12 hours following treatment. Mortality was higher when early 3rd- or 2nd-instar larvae were used, but lower when 4th-instar larvae were treated. MON-0585 was routinely used as a reference compound in treating larvae obtained from any single batch. Since results obtained in long term toxicological studies of inhibitors of oxidative systems depend so much upon the conditions employed (cf below), it is suggested that the use of MON-0585 as a reference compound be continued.

Mortality often was high within the 24-hour period following treatment. For example, larvae treated with 53, 74, and 76 at 0.1 ppm visibly responded to the test compound within 30 minutes. The larvae became agitated and frequently collided with each other.

Larvae that failed to respond to stimuli of light, sound and motion after 24 hours were often normal after 48 hours. Most of the catechols and quinones (Tables 5 and 6) showed this type of response. As an example, 7 out of 20 larvae treated with catechol (54) at 1 ppm were moribund and considered "dead" at 24 hours, but only 2 were dead at 48 and 72 hours after treatment. However, 80% of the larvae were dead before the end of the bioassay period (21 days). A similar effect has been observed for phenothiazine[12] a compound which also is a free radical inhibitor.

Apart from 24-hour mortality, most larval deaths occurred immediately after ecdysis or after an unduly long larval duration. Larvae treated with catechols and 2,3-naphthalenediols (Tables 5 and 6) often remained attached to the terminal appendages of the moult from the previous instar. These larvae drowned slowly. Death in the pupal stages also commonly occurred immediately after pupation or in the late pharate adult stage. The latter were identified by their "sooty black" color.

Adults frequently failed to emerge fully after ecdysis. The degree of emergence varied from a simple split along the middle line of the cephalo-thorax to emergence of all parts except the abdomen and wing tips. Partially emerged adults remained alive but were unable to escape.

The results described above parallel results obtained from derivatives of farnesoic acid[13] and from MON-0585[11]. It appears that inhibitors of oxidative systems in general produce apparent JH effects. This may be attributed to interference of the metabolism of endogenous JH, but interference with other oxidative processes is also possible.

Several of the compounds tested were significantly more effective as mosquito larvicides than MON-0585. The most effective compounds were 43, 45, 53, 61, 74, 76, 80, 81, and 82. These materials were equal to or more effective than MON-0585 under similar test conditions. It is noteworthy that each of the various classes of compounds studied is represented by at least one compound with high activity. Of some significance is the fact that two of the more active compounds, 74 and 76, are of plant origin. The most effective compound was 4-nitro-5-phenoxycatechol (53) where significant activity was observed as low as 0.02 ppm. The corresponding methylenedioxy derivative (45) also was effective. The latter, as described in the preceding section, were found to be one of the most powerful carbamate synergists ever examined in this laboratory.

It should be noted that the toxicity obtained repeatedly in this work for MON-0585 is below the $LD_{50}$ value of 0.05 ppm quoted for the 3rd-instar *Aedes aegypti* larvae by Sacher[11]. In our study treatment of 20 *Culex pipiens* larvae in 100 ml of water at a concentration of 0.05 ppm resulted in only 25% mortality (average of 16 determinations). However, treatment of 20 larvae in 40 ml of water at a concentration of 0.05 ppm resulted in 86% mortality (average of 16 determinations). Emergence of controls at the two different volumes of 40 and 100 ml were identical. It appears, therefore, that the effect of MON-0585 is increased by a "crowding" factor. This result emphasizes the importance of using a reference compound in all long-term toxicological studies of mosquito larvae.

Conclusion - A variety of substituted 1,3-benzodioxoles, catechols, and quinones, all materials which affect free radical processes, were quite effective larvicides, showing activity comparable to or greater than that demonstrated by MON-0585. These compounds appeared to inhibit the development of larvae into pupae or adults. Symptoms of intoxication paralleled those obtained from the treatment of mosquito larvae with farnesoic acid (a JH mimic) or MON-0585.

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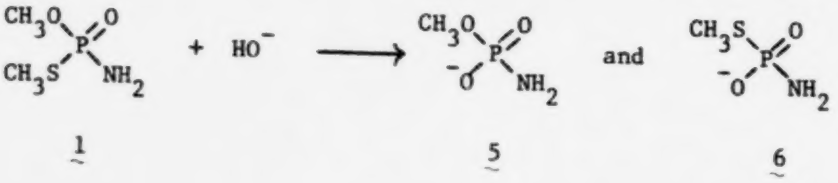
## H. STUDIES ON CHEMICAL REACTIONS INVOLVING CARBAMATE AND ORGANOPHOSPHORUS ESTERS

During the project period several investigations were conducted on some of the fundamental aspects of reactions involving phosphoramidothioate and carbamate esters. The results are summarized below.

### 1. Alkaline Hydrolysis of Phosphoramidothioate Esters

Introduction - *O*,*S*-Dimethyl phosphoramidothioate (Monitor or 1) is a relatively simple organophosphorus ester which has proved to be a highly useful insecticide. In an earlier investigation[1] on the mode of action of 1 and related esters it was assumed that the alkylthiolate moiety was released when the cholinesterase enzyme was inhibited by the phosphoramidothioate ester. Subsequently, however, examination of the reaction between 1 and hydroxide ion showed that methylthiolate ion was not always the major product but methoxide also was liberated, the relative amounts depending on the conditions of the reaction. Because of the possible connection between alkaline hydrolysis rates and anticholinesterase activity, an examination of the alkaline hydrolysis of 1, its *N*-methyl (2) and *N*,*N*-dimethyl (3) analogs was conducted. Product and kinetic analysis were undertaken to sort out the various individual reactions and to assess quantitatively their relative importance in the overall hydrolysis reaction. Particular attention was given to the effect of sequential substitution of methyl groups on the nitrogen atom and of solvent on the specific rates of P-O and P-S bond cleavage.

Products of Alkaline Hydrolysis - Pmr spectra of the products obtained from the hydrolysis of *O*-methyl *S*-methyl phosphoramidothioate (1) with equimolar amounts of potassium hydroxide in water and in 50% aqueous acetone showed that two monoanionic products were obtained, one by P-S cleavage giving *O*-methyl phosphoramidate anion (5) and the other by P-O cleavage giving *S*-methyl phosphoramidothioate anion (6). In water the major product obtained was 6 since analysis of the pmr integrals for P-$OCH_3$ protons and P-$SCH_3$ protons showed a

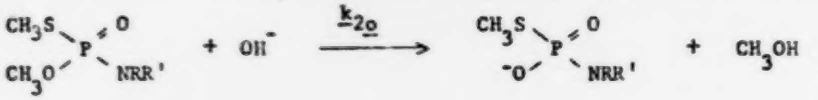


(1)

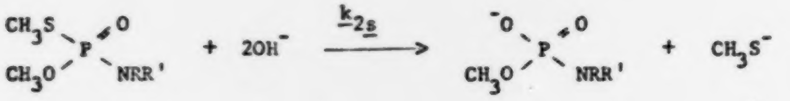
ratio of 5/6 of 1/4.5. In 50% aqueous acetone, however, 5 was the major product, and the ratio of 5/6 in this case was 5.1/1. Support for the ratio of products obtained by integration of pmr spectra was provided by glc analysis after remethylation of the mixture of 5 and 6 with diazomethane. Remethylation of 5 and 6 gave dimethyl phosphoramidate (4), retention time 1.75 min, and 1, retention time 3.50 min, respectively, and the ratio of these products was virtually identical to the ratio of 5/6 obtained by proton integration. Confirmation of product ratios by glc, therefore, allowed the use of pmr as the major means of product analysis.

Data for product analysis by pmr after alkaline hydrolysis of 1 in a variety of solvent systems are given in Table 1. The results indicate that the solvent strongly influences the relative percentages of 5 and 6 produced and, in the case of the methanol-water system, increasingly larger amounts of P-O bond cleavage were obtained with increasing amounts of water; e.g. 5/6 in absolute methanol was 93/7 compared to 18/82 in water.

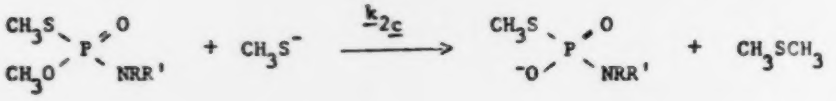
When 1 was treated with an equimolar amount of potassium hydroxide in absolute ethanol or propanol, the major product was neither 5, nor 6 but was the potassium salt of O-ethyl and O-propyl phosphoramidic acid, respectively. Further, distillation of the reaction mixture containing n-propanol or ethanol as a solvent gave a low boiling fraction (37-40°) which was identified by glc and pmr as dimethyl sulfide. Dimethyl sulfide also was isolated by the same procedure in varying quantities after the treatment of 1, O-methyl S-methyl N-methylphosphoramidothioate (2) and O-methyl S-methyl N,N-dimethylphosphoramidothioate (3) with potassium hydroxide in water (Table 2). Dimethyl sulfide apparently is formed from the reaction between methylthiolate anion, produced by P-S bond cleavage, and the starting ester. Therefore, the reactions involved in the decomposition of (1) and related esters by hydroxide ion may be depicted as follows.



(2)



(3)



(4)

1 R,R' = H

2 R = H,R' = $CH_3$

3 R,R' = $CH_3$

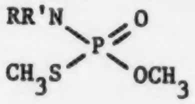
$k_{2o}$, $k_{2s}$ and $k_{2c}$ are the specific second-order rate constants for

Table 1. SALT DISTRIBUTION IN ALKALINE HYDROLYSIS OF O-METHYL S-METHYL PHOSPHORAMIDOTHIOATE IN VARIOUS ORGANIC-WATER SOLVENT MIXTURES AT 23°

| % Organic Solvent in aqueous mixture (v/v) | | % Salt Containing | |
|---|---|---|---|
| | | P-OCH$_3$ | P-SCH$_3$ |
| methanol | 100 | 93 | 7 |
| | 95 | 86 | 14 |
| | 90 | 79 | 21 |
| | 85 | 76 | 24 |
| | 80 | 67 | 33 |
| | 75 | 63 | 37 |
| | 65 | 50 | 50 |
| | 50 | 40 | 60 |
| | 0 | 18 | 82 |
| acetone | 50 | 83 | 17 |
| mesityloxide 10, ethanol | 30 | 33 | 67 |
| propionaldehyde 50, ethanol | 25 | 80 | 20 |
| formaldehyde[a] 30 | | 100 | - |
| acetophenone 20, ethanol 60 | | 25 | 75 |
| acetonitrile 50 | | 23 | 77 |
| 2-butanone 45, ethanol 10 | | 60 | 40 |
| ethanol 100 | | 7.5 | 12.5 |
| | | P-OC$_2$H$_5$ 80% | |
| propanol 100 | | 10 | 22.5 |
| | | P-OC$_3$H$_7$ | |
| benzaldehyde 25, ethanol 20 | | 83 | 17 |

[a] Extensive polymerization occurred.

Table 2. PRODUCT DISTRIBUTION AFTER HYDROLYSIS OF PHOSPHORAMIDOTHIOATES BY AQUEOUS POTASSIUM HYDROXIDE IN A SEALED AMPOULE



| R | R' | Temp °C | Reaction time (hr) | % Salt containing | | % MeOH | % $CH_3SCH_3$ |
|---|---|---|---|---|---|---|---|
| | | | | P-$OCH_3$ | P-$SCH_3$ | | |
| H | H | 23 | 4 | 18 | 82 | 75 | 10 |
| H | $CH_3$ | 23 | 16 | 52 | 48 | 30 | 19 |
| $CH_3$ | $CH_3$ | 23 | 96 | 50 | 50 | 13-15[a] | -[b] |
| $CH_3$ | $CH_3$ | 96 | 14 | 42 | 58 | 24 | 32 |

---

[a] Reaction mixture was distilled to obtain methanol in high concentration for glc.

[b] Reaction at atmospheric pressure, dimethylsulfide was not determined.

P-O, P-S and C-O bond cleavage, respectively.

Evidently, *S*-methyl phosphoramidothioate anion (6) may be produced in two ways, from replacement by hydroxide ion according to equation (2) or from *O*-demethylation by methylthiolate ion according to equation (4). The extent of 6 (or its *N*-methyl and *N*,*N*-dimethyl equivalent) formed by replacement of methoxide by hydroxide ion was determined by glc analysis for methanol in the reaction mixture. Quantitative data showing the relative amounts of the various products produced after treatment of 1, 2, and 3 by equimolar aqueous potassium hydroxide are presented in Table 2. The results indicate that the *O*-demethylation reaction to produce 6 or its *N*-alkyl derivatives becomes increasingly important with sequential methylation of nitrogen.

Finally, in order to determine whether potassium hydroxide treatment of 1 produced methanol by P=O or C-O bond cleavage, the reaction was carried out in water enriched with $H_2O^{18}$. The liberated methanol was examined by mass spectrometry and no significant $O^{18}$-incorporation was found, indicating that hydrolysis occurred by P-O bond cleavage.

*Kinetics of Hydrolysis of O-Methyl S-Methyl Phosphoramidothioate* - Under conditions of high concentrations of 1 compared to hydroxide, the value for the overall pseudo first-order rate constant $k_1$ were obtained by following hydroxide ion consumption from estimation of the change in pH. Under these conditions equation 4 may be omitted in the kinetic analysis of 1 since hydroxide ion is not involved in this equation. Also, $k_1 = k_{1o} + ak_{1s}$ where $k_{1o}$ and $k_{1s}$ are the pseudo first-order constants for P-O and P-S cleavage, respectively. The value of the coefficient $a$ depends on the pH of the solution, i.e. $a$ is 2 at pH substantially greater than the $pK_a$ of methanethiol (10.7) and $a$ is 1 at pH lower than 10.7. From the slope of a plot between pH and time, the value for the overall pseudo first-order constant $k_1$ at 27° was calculated to be 0.96 $min^{-1}$ and 9.6 $M^{-1}$ $min^{-1}$ (1 was 0.1 M), respectively.

The value of $k_{2o}$, the second-order constant for P-O bond cleavage was determined directly by following methanol production under second-order conditions of 1 and hydroxide ion. The plot of the data was reasonably linear up to about 65% reaction and $k_{2o}$ was calculated to be 8.4 $M^{-1}$ $min^{-1}$ at 27°. From the relationship $k_2 = k_{2o} + 2k_{2s} = 9.6$ $M^{-1}$ $min^{-1}$, $k_{2s}$ was calculated to be 0.6$M^{-1}$ $min^{-1}$. Values for $k_{2o}$ and $k_{2s}$ are given in Table 3.

*Effect of Acetone on Hydrolysis of 1.* - The effect of acetone on the values of the pseudo first-order constants $k'_{1o}$ and $k'_{1s}$ under conditions of constant pH for P-O and P-S cleavage is shown in Table 4. Because of interference by acetone in the glc analysis of methanol, it was not possible to determine $k'_{1o}$ directly in solvents containing acetone. Therefore, $k'_{1o}$ was calculated from the values of $k'_1$ (overall pseudo first-order constant at constant pH) and $k'_{1s}$ determined

Table 3. SECOND-ORDER RATE CONSTANTS FOR P-O ($k_{2o}$) AND P-S ($k_{2s}$) CLEAVAGE OF PHOSPHORAMIDOTHIOATES IN AQUEOUS POTASSIUM HYDROXIDE AT 27°

| | $k_{2o}$ ($M^{-1}$ min$^{-1}$) | $k_{2s}$ ($M^{-1}$ min$^{-1}$) |
|---|---|---|
| $(CH_3O)(CH_3S)P(O)NH_2$ | 8.4 | 0.6 |
| $(CH_3O)(CH_3S)P(O)NHCH_3$ | $1.0 \times 10^{-3}$ | $4.4 \times 10^{-2}$ |
| $(CH_3O)(CH_3S)P(O)N(CH_3)_2$ | - | $1.5 \times 10^{-4}$ |

Table 4. PSEUDO FIRST-ORDER RATE CONSTANTS IN AQUEOUS ACETONE FOR THE ALKALINE HYDROLYSIS OF O-METHYL S-METHYL PHOSPHORAMIDOTHIOATE AT pH 10, $\mu = 0.2$ M

| % Acetone (v/v) | $k'_{1s} \times 10^3$ (min$^{-1}$) | $k'_{1o} \times 10^3$ (min$^{-1}$) | Calcd from $CH_3SH$ release ($t_{½}$) | Calcd from $HO^-$ consumption |
|---|---|---|---|---|
| 0 | - | - | - | 3.0 |
| 10 | 1.6 | 5.0 | 6.9 | - |
| 20 | 3.1 | 5.5 | 8.6 | - |
| 30 | 4.2 | 5.9 | 10.6 | 10.1 |
| 40 | 6.2 | 6.0 | 12.5 | 12.2 |

at pH 10.0 in aqueous solvents containing 0-40% acetone and sodium carbonate as the buffer. $k'_{1s}$ was calculated from the relationship below by following methanethiol formation where $[A_o]$ is the initial

$$\frac{[CH_3SH]}{[A_o]} = \frac{k'_{1s}}{k'_1} (1 - e^{-k'_1 t}) \qquad (5)$$

concentration of **1**. A plot of $[CH_3SH]/A_o$ against $(1 - e^{-k'_1 t})$ gave a linear relationship and $k'_{1s}$ was calculated from the slope. The value of $k'_1$ was determined from the amount of base needed to maintain a pH of 10.0 using a micrometer driven syringe containing standardized aqueous potassium hydroxide. $k'_1$ also was calculated from the relationship $t_{\frac{1}{2}} = \ln 2/k'_1$ which follows from equation 5 where $t_{\frac{1}{2}}$ is the time released to liberate one-half of the total amount of methanethiol released. The data in Table 4 show good agreement between the values of $k'_1$ obtained by estimation of potassium hydroxide consumption and that calculated from the $t_{\frac{1}{2}}$ value for methanethiol release.

Hydrolysis of *O*-Methyl *S*-Methyl *N*-Methylphosphoramidothioate (**2**) - The overall rate of reaction between *O*-methyl *S*-methyl *N*-methylphosphoramidothioate (**2**) and hydroxide ion determined by estimating change in pH was approximately 110-fold slower than **1**, with a pseudo first-order rate (excess of **2**) constant $k_1$ of $4.45 \times 10^{-2}$ $min^{-1}$ and a second-order rate constant $k_2$ of $8.9 \times 10^{-2}$ $M^{-1}$ $min^{-1}$. Because of the slow rate of reaction between **2** and hydroxide ion, loss of **2** by dealkylation (equation 3) was significant and it was possible to determine $k_{1c}$ (the pseudo first-order rate constant for C-O cleavage) as well as $k_{1o}$ and $k_{1s}$. Kinetic analysis was accomplished by imposing again, as in the case of **1**, pseudo first-order conditions, i.e. high concentrations of **2** relative to hydroxide ion. Under these conditions, equations 2, 3, and 4 may be designated as follows.

$$A + B \xrightarrow{k_{1o}} C \qquad (6)$$

$$A + B \xrightarrow{k_{1s}} D \qquad (7)$$

$$A + D \xrightarrow{k_{1c}} G \qquad (8)$$

where A = 2, B = hydroxide ion, C = methanol, D = methylthiolate ion, and G = dimethyl sulfide. From equations 6, 7, and 8, and since A is constant, 9 and 10 may be obtained.

$$\frac{-d[B]}{dt} = k_1[B] = k_{1o}[B] + 2k_{1s}[B] \qquad (9)$$

$$\frac{d[D]}{dt} = k_{1s}[B] - k_{1c}[D] \qquad (10)$$

Since $B = B_o\, e^{-k_1 t}$, where $B_o$ is the initial hydroxide ion concentration, equation 10 becomes 11.

$$\frac{d[D]}{dt} = k_{1s} B_o\, e^{-k_1 t} - k_{1c}[D] \qquad (11)$$

This first-order differential equation has the following solution.

$$[D] = \frac{k_{1s}[B_o]}{k_{1c} - k_1} \left(e^{-k_1 t} - e^{-k_{1c} t}\right) \qquad (12)$$

When $k_{1c}$ is small compared to $k_{1s}$, integration of equation 11 results in 13.

$$\frac{[D]}{[B_o]} = \frac{k_{1s}}{k_1} \left(1 - e^{-k_1 t}\right) \qquad (13)$$

This equation is similar to equation 5 where $B_o$ (initial hydroxide ion concentration) is substituted for $A_o$ (phosphoramidothioate concentration).

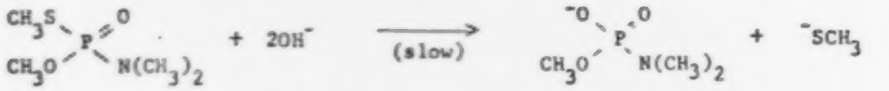
The plot of $[CH_3S^-]/[OH_o^-]$ vs $(1 - e^{-k_1 t})$ gave the expected linearity over the major portion of the reaction, from which $k_{1s}$ at 27° was calculated as $2.20 \times 10^{-2}$ min$^{-1}$. From the relation $k_1 = k_{1o} + 2k_{1s} = 4.45 \times 10^{-2}$ min$^{-1}$ the value of $k_{1o}$ was calculated as $5 \times 10^{-4}$ min$^{-1}$. The corresponding second-order rate constants, $k_{2o}$ and $k_{2s}$ given in Table 3 were obtained by dividing the values of $k_{1o}$ and $k_{1s}$ by 0.5 M (initial concn of 2).

The value of $k_{1c}$ was calculated by using equation 12 with the aid of a computer. By setting the value of $k_1$ to $4.45 \times 10^{-2}$ min$^{-1}$, estimated values of $k_{1s}$ and $k_{1c}$ were substituted in equation 12 until the calculated values of methylthiolate ion concentration at different time intervals coincided with the experimental values. Best fit of the data was obtained when $k_{1s}$ was $2.2 \times 10^{-2}$ min$^{-1}$ and

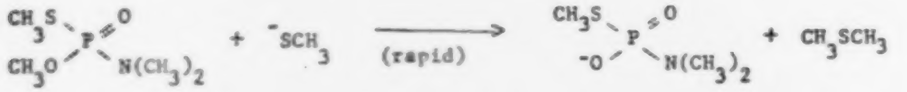
$k_{1c}$ was 3.6 X $10^{-3}$ min $^{-1}$.

Hydrolysis of O-Methyl S-Methyl N,N-Dimethylphosphoramidothioate (3) - The rate of hydrolysis of 3 was much slower than 2 under identical first-order conditions of excess phosphoramidothioate over base with a pseudo first-order constant $k_1$ for the disappearance of hydroxide ion of 1.4 X $10^{-4}$ min$^{-1}$. No methanethiol was detectable at any time during the course of the reaction, indicating that the rate of reaction between methanethiolate ion and 3 to form dimethyl sulfide was considerably faster than the initial reaction between hydroxide ion and 3. $k_{1c}$, the pseudo first-order rate constant for methyl-oxygen bond cleavage for 2 and 3 under the same condition should be similar, and based on the value of $k_{1c}$ = 3.6 X $10^{-3}$ min$^{-1}$ obtained for 2, the dealkylation reaction for 3 should be approximately 25-fold faster than the initial reaction.

Analysis of products after reaction between 3 and potassium hydroxide showed that P-O cleavage was much slower than P-S cleavage (23°) and this coupled with the above information gives the order $k_{1c} > k_{1s} \gg k_{1o}$. By making the assumption that $k_{1o}$ is negligibly small compared to $k_{1s}$, the kinetics of the reaction may be approximated to a second-order situation with the provision that 2 moles of 3 are consumed (one mole by P-S cleavage in the rate determining step and one mole by a rapid dealkylation reaction) with 2 moles of hydroxide ion (one mole for P-S cleavage and 1 mole for rapid neutralization of the resulting phosphoramidic acid) according to the following equations.



(14)



(15)

By using equimolar amounts of 3 and hydroxide ion the value for the second-order rate constant $k_{2s}$ for P-S cleavage may be determined from equation 16 where $[OH^-]$ and $[OH_o^-]$ are the concentrations of hydroxide ion at time t and time zero, respectively. A plot of

$$1/[OH^-] = 1/[OH_o^-] + 2k_{2s}t \qquad (16)$$

$1/[OH^-]$ against time from data obtained in a separate experiment using equimolar concentrations of 3 and hydroxide ion gave an excellent straight line over a 45% reaction range, a finding which provided support for the assumptions made in the kinetic approach. From the plot, $\underline{k}_{2s}$ was found to be $1.5 \times 10^{-4}$ $\underline{M}^{-1}$ $\text{min}^{-1}$ ($27^{\circ}$).

The value of $\underline{k}_1$ ($1.4 \times 10^{-4}$ $\text{min}^{-1}$) obtained under the pseudo first-order condition of excess phosphoramidothioate (3 was 0.5 $\underline{M}$) also is equal to $2\underline{k}_{1s}$ since $\underline{k}_{1o}$ is negligible. From this relationship also $\underline{k}_{2s}$ may be calculated as $1.4 \times 10^{-4}$ $\underline{M}^{-1}$ $\text{min}^{-1}$, in good agreement with the value obtained under second-order conditions.

Analysis of Data - Because of the known greater lability of the P-S bond, the hydrolysis of 1 in aqueous potassium hydroxide by predominantly P-O bond rather than P-S bond cleavage was unexpected. The ionizing capacity of the solvent evidently plays a dominant role in establishing the direction in which 1 is hydrolyzed by potassium hydroxide. The influence of solvent on the reaction is illustrated in Figure 1 which shows the relation between Grunwald-Winstein Y values[2] for water-methanol and the log of the ratio of 6 to 5. Although the product ratio in two parallel second-order reactions at any time is equal to the ratio of rate constants, the value of 6/5 may only be considered as an approximation of the relative rate constants for P-O and P-S cleavage since the same salt is obtained by O-demethylation and P-O cleavage. Figure 1 shows, however, that alkaline hydrolysis of 1 by P-O bond cleavage is favored in solvents of greater ionizing capacity. In less polar solvents P-S bond cleavage predominates, suggesting that the two competing reactions (P-O and P-S cleavage) occur by different mechanisms.

Table 4 shows the effect of increasing amounts of acetone on the pseudo first-order rate constants for P-O ($\underline{k}'_{1o}$) and P-S ($\underline{k}'_{1s}$) cleavage of 1 at pH 10.0. Figure 2 gives the relation between these constants and Grunwald-Winstein Y values for the relevant acetone-water mixtures. Although the relationship is clearly not linear, it does focus on the far greater dependency of $\underline{k}'_{1s}$ on solvent polarity, e.g. $\underline{k}'_{1s}$ increases almost 4-fold from 10% to 40% acetone while $\underline{k}'_{1o}$ remains virtually constant. Thus, in increasing a polar solvent P-S cleavage becomes increasingly important, resulting eventually in a changeover in ratio of products.

The values for the specific second-order rate constants for P-O ($\underline{k}_{2o}$) and P-S ($\underline{k}_{2s}$) bond cleavage for 1, 2, and 3 in aqueous potassium hydroxide at $27^{\circ}$ in Table 3 show that there is a marked decrease in both $\underline{k}_{2o}$ and $\underline{k}_{2s}$ with sequential substitution of amido protons by methyl. In addition, the relative rates for P-O and P-S cleavage also decreased, e.g. $\underline{k}_{2o}/\underline{k}_{2s}$ was 14 for 1, 0.023 for 2 and presumably much smaller for 3. Thus, 1 was hydrolyzed predominantly by P-O cleavage while P-S cleavage predominated with 2 and 3.

A plausible mechanism in which two different processes are taking place simultaneously may be suggested from the data, process (a)

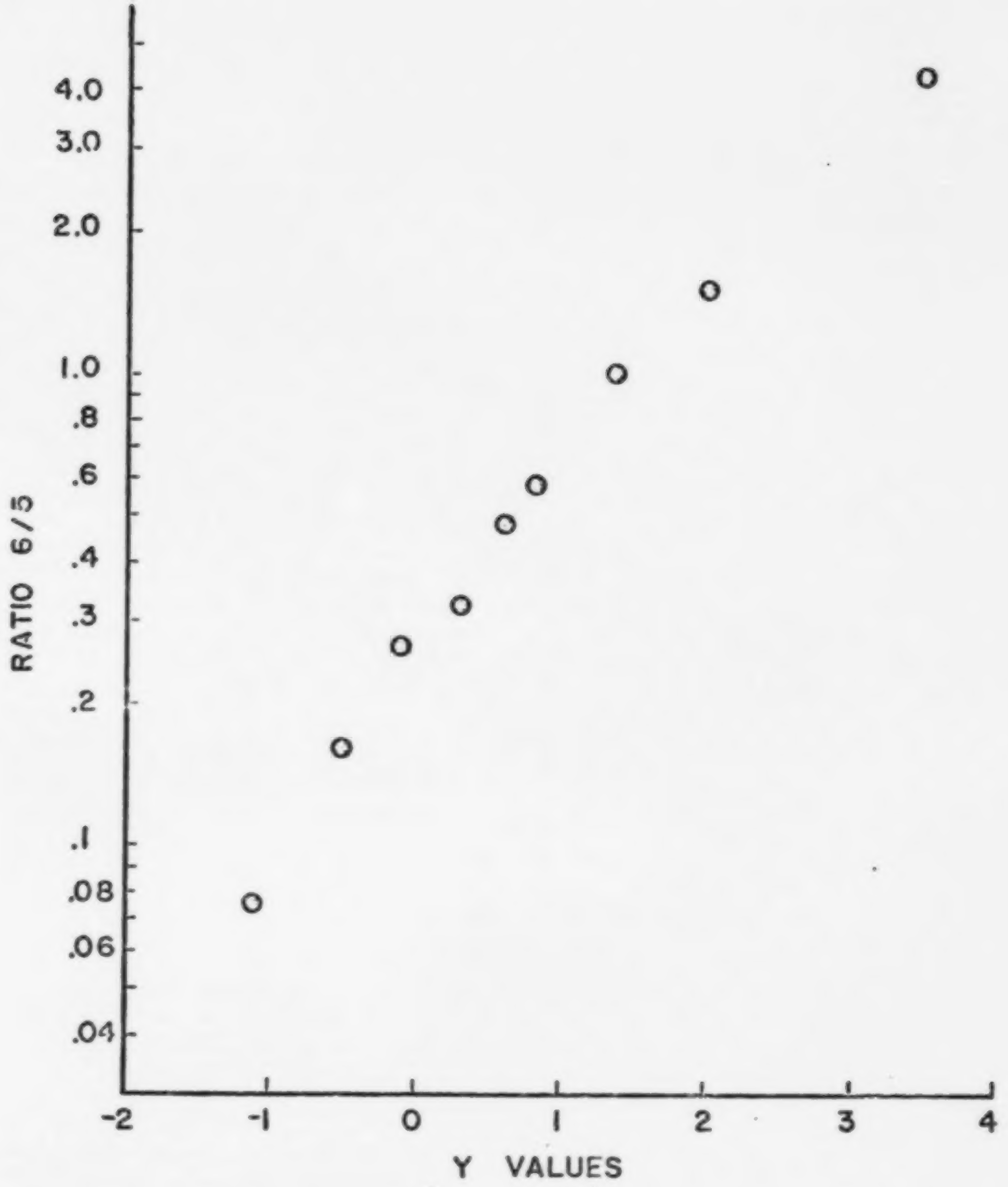


Figure 1. Relation between Grunwald-Winstein Y values and the log of the ratio of potassium S-methyl phosphoramidothioate (6) to O-methyl phosphoramidate (5).

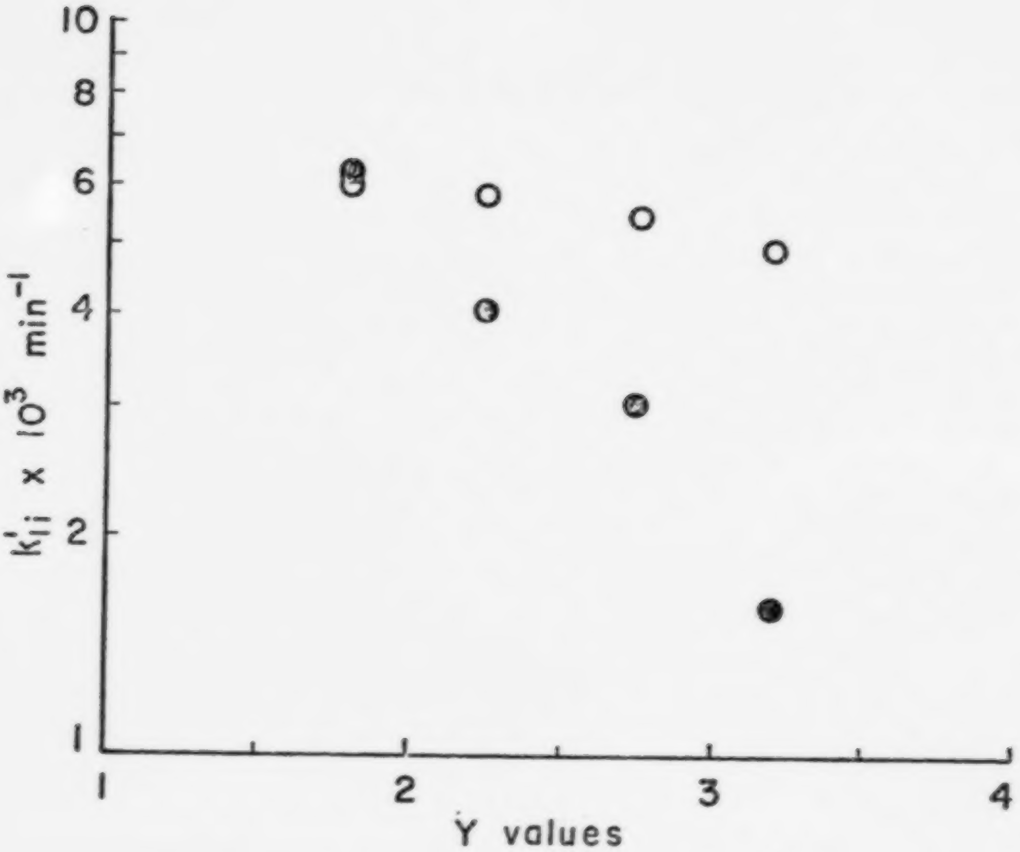
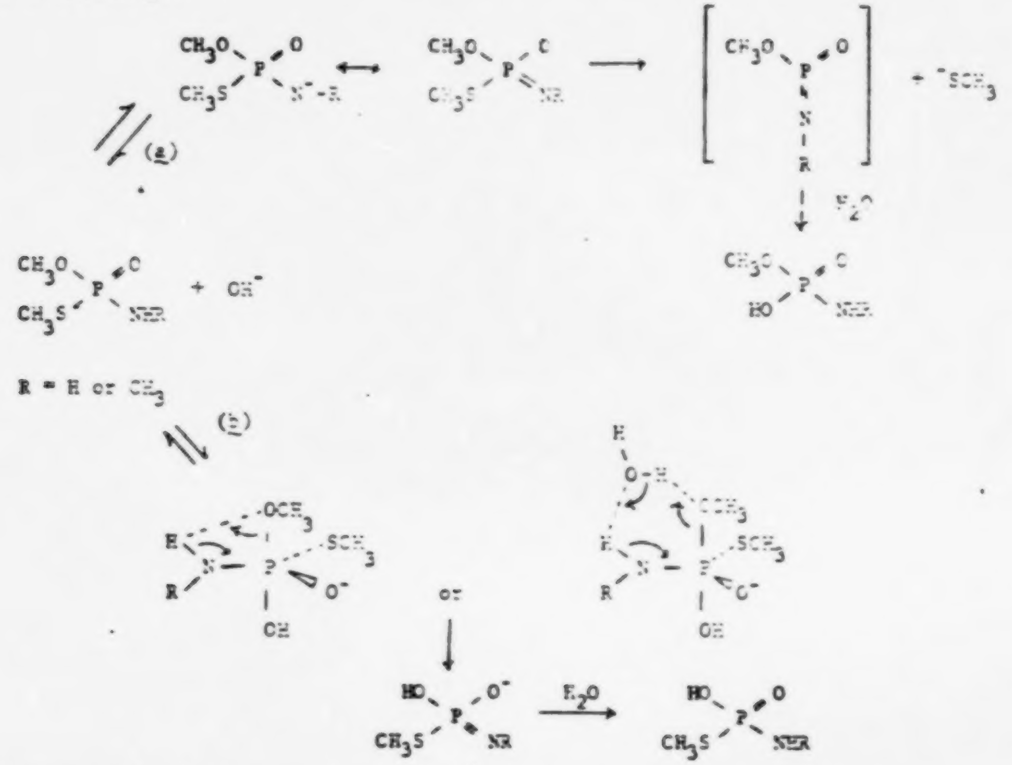
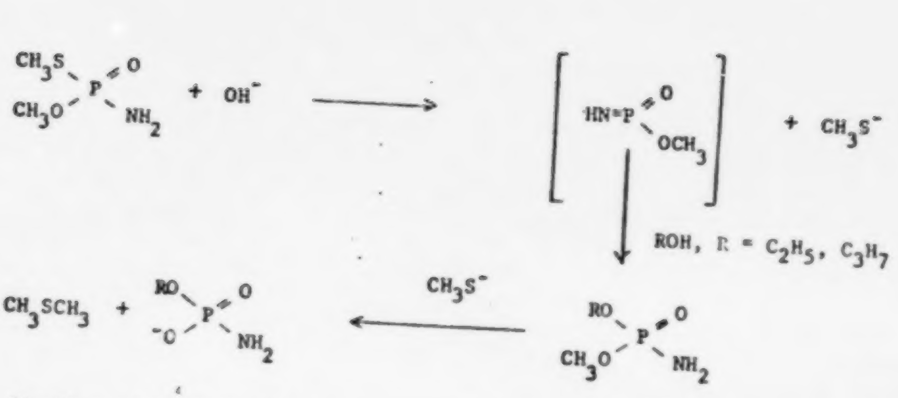


Figure 2. Relationship between $\underline{k}_{1\underline{s}}$ (●) and $\underline{k}_{1\underline{o}}$ (○) and Grunwald-Winstein Y values for the alkaline hydrolysis of O-methyl S-methyl phosphoramidothioate in aqueous acetone.

which involves hydroxide ion attack on the amido proton, leading to the phosphoramidothioate anion which decomposes in a rate determining step to give P-S cleavage, and process (**b**) which involves attack of hydroxide ion on the phosphorus atom, eventually resulting in P-O cleavage.



Process **a** leading to P-S cleavage and phosphorimidate formation is analogous to the mechanism proposed for base catalyzed hydrolysis of phosphoramidic chlorides. Direct support for this mechanism is found in results obtained from the analysis of products after base catalyzed hydrolysis of **1** in absolute ethanol and propanol. The observation that **1** reacts with potassium hydroxide in absolute ethanol or propanol to give as major products dimethyl sulfide and the potassium salt of ethyl and propyl phosphoramidic acid, respectively, strongly indicates that this reaction proceeds through a metaphosphorimidate. In ethanol or propanol the intermediate is solvolyzed to give ethyl or propyl methyl phosphoramidate which is in turn demethylated by methylthiolate anion to produce the respective products as shown below.



The finding that P-O cleavage predominates in highly polar solvents suggests that this reaction proceeds by a process involving hydroxide ion attack on the phosphorus atom, either by a concerted $S_N2(P)$ or by an addition-elimination reaction. Although a concerted $S_N2$ reaction may lead to P-O cleavage, it does not account for the preponderance of P-O over P-S cleavage, particularly since methylthiolate ion is a superior leaving group compared to methoxide ion. Thus, process b leading to P-O cleavage probably involves, at least in the case of 1 and 2, the addition of the hydroxide ion to the phosphoramidothioate followed by a rapid elimination of the alkoxy group rather than a concerted $S_N2$ type reaction. The addition step is expected to lead to a trigonal bipyramidal intermediate and from preference rules[3] the methoxy and hydroxy groups should occupy apical positions. With the methoxy group in an apical position, its departure may be assisted by the nitrogen proton either directly or indirectly by the intervention of a water molecule as indicated in the mechanism given above. The addition-elimination mechanism explains why P-O cleavage takes place with 1 and 2 but does not occur with 3 where the nitrogen atom is fully substituted with methyl groups. The exclusive cleavage of the P-S bond in 3 probably occurs by a concerted $S_N2$ mechanism in which the best leaving group, i.e. methylthiolate, is displaced by hydroxide ion. This is the usual type of substitution reaction with most triesters of phosphoric acid. The fact that the hydrolysis of 3 by P-S cleavage is approximately 3 X $10^4$ times slower than that of 1 suggests different mechanisms for the hydrolysis of 1 and 3.

Conclusion - The results indicate that solvent polarity has a profound effect on the direction in which phosphoramidothioate esters hydrolyze under alkaline conditions. In aqueous potassium hydroxide O,S-dimethyl phosphoramidothioate is hydrolyzed by P-O cleavage as the major route while in less polar solvents P-S cleavage predominates.

Both rate constants and the relative rates of P-O/P-S bond cleavage decreased markedly with sequential substitution of the amido protons with methyl groups, and the N,N-dimethyl derivative hydrolyzed virtually exclusively by P-S bond cleavage but at a rate some $10^4$ times slower than P-S cleavage in unsubstituted phosphoramidothioate. Exclusive P-S bond cleavage in the N,N-dimethylphosphoramidothioate evidently occurs by a normal concerted $S_N2$ reaction in which the best leaving group departs. In phosphoramidothioates containing at least one amido proton the results are rationalized in terms of two competing preocesses, an addition-elimination reaction on phosphorus leading to P-O bond cleavage and an elimination reaction involving the amido proton to give P-S bond cleavage.

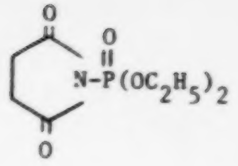
The question remains, what is the leaving group (methoxide or thiomethylate), when cholinesterase is inhibited by Monitor or a related phosphoramidothioate ester? The fact that P-O cleavage predominates in an aqueous environment would suggest that methoxide is the leaving group in the inhibition process. However, because of the presence of lipophilic sites on the enzyme it is not possible to rule out thiomethylate as the leaving group. The chemistry and mode of action of Monitor and related phosphoramidothioate esters deserve further study.

## 2. Reaction of Trialkyl Phosphites with Haloamides

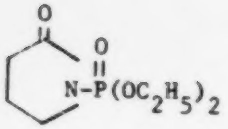
Introduction - Synthetic routes to insecticidally active O,S-dialkyl N-acylphosphoramidothioates are multistep and often result in poor yields. Because of the need to prepare a radiolabel of the insecticide Orthene (O,S-dimethyl N-actylphosphoramidothioate) for metabolism studies, alternative methods for a convenient synthesis of this compound were investigated. In particular, the reaction between N-bromoacetamide and trimethyl phosphorothioate was examined. In all cases, no Orthene could be isolated although the starting materials were consumed and alkyl halides were evolved. In an attempt to understand this reaction, the reaction between triethyl phosphite and N-haloamides was investigated.

Product Analysis - The reaction between trialkyl phosphites and the following N-haloamides was examined:
N-bromoacetamide (7), N-chlorobenzamide (8), N-chlorosuccimide (9),
N-bromosuccimide (10), N-bromo-2-pyrrolidinone (11),
N-chloroacetamide (12), N-chloro-N-methylacetamide (13),
and N-bromobenzamide (14)

The cyclic haloamides (9-11) reacted with 1 equivalent of triethyl phosphite to give ethyl halide and the expected Arbuzov products 15 or 16. The product 15, which was the same whether prepared from



15

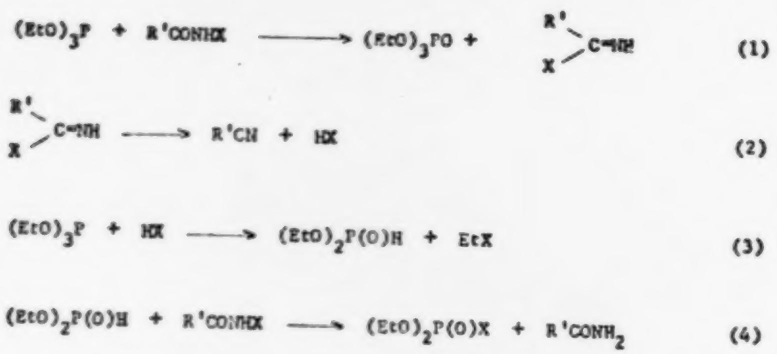


16

9 or 10, was identical to that reported previously.[4]

The acyclic primary haloamides (7, 8, 12, and 14) did not give the expected Arbuzov products but instead reacted to give products (Table 5) consistent with Scheme I.

Scheme I (Square brackets are used to indicate intermediates that were never isolated)



$$(EtO)_3P + R'CONHX \longrightarrow (EtO)_3PO + R'(X)C{=}NH \quad (1)$$

$$R'(X)C{=}NH \longrightarrow R'CN + HX \quad (2)$$

$$(EtO)_3P + HX \longrightarrow (EtO)_2P(O)H + EtX \quad (3)$$

$$(EtO)_2P(O)H + R'CONHX \longrightarrow (EtO)_2P(O)X + R'CONH_2 \quad (4)$$

$R' = CH_3, C_6H_5$

$X = Cl, Br$

When N-chloro-N-methylacetamide (13) and trialkyl phosphite were reacted, the only products were the trialkyl phosphate and N-methylacetimidoyl chloride, analogous to step 1 in the scheme. For the primary haloamides, Scheme I is supported by the following evidence.

1. Reaction of one equivalent of primary haloamide with one equivalent of triethyl phosphite led to the formation of approximately one-half equivalent of ethyl halide, nitrile, amide, triethyl phosphate, and diethyl halophosphate (cf Table 5).

2. The reaction was exothermic until almost two equivalents of triethyl phosphite had been added. At this point, no triethyl phosphite could be isolated when it was introduced rapidly.

Table 5. PRODUCTS OBTAINED FROM THE REACTION BETWEEN N-HALOAMIDES AND TRIETHYL PHOSPHITE AT 25°

| Haloamide | Liquid vehicle | Moles of reagents | | Moles of products | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Haloamide | Phosphite | RCN | $RCONH_2$ | EtX | $(EtO)_2P(O)X$ | $(EtO)_2P(O)H$ | $(EtO)_3P(O)$ |
| 7 | toluene | 0.5 | 1.0 | 0.46 | <0.04 | 0.45 | ≈0 | 0.36 | 0.41 |
| 8 | $CCl_4$ | 0.5 | 1.0 | 0.44 | 0.02 | 0.25 | 0.07 | 0.32 | 0.45 |
| 9 | ether | 1.0 | 1.0 | 0.34 | 0.42 | - | 0.32 | 0.02 | 0.47 |
| 10 | benzene | 1.0 | 1.0 | 0.41 | 0.48 | 0.48 | 0.36 | <0.01 | 0.41 |
| 11 | benzene | 1.0 | 1.0 | 0.47 | 0.48 | 0.50 | 0.42 | ≈0 | 0.36 |
| 12 | $CCl_4$ | 0.5 | 1.0 | 0.48 | <0.02 | 0.50 | 0.03 | 0.36 | 0.36 |
| 13 | $CCl_4$ | 1.0 | 1.0 | 0.48 | 0.44 | 0.50 | 0.37 | 0.05 | 0.46 |
| 14 | ether | 1.0 | 1.0 | 0.44 | 0.46 | 0.37 | 0.43 | 0.06 | 0.43 |

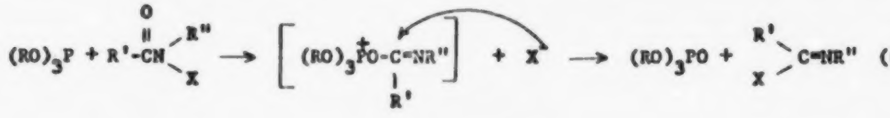
3. Addition of two equivalents of triethyl phosphite to one equivalent of primary haloamide gave in good yields the products indicated in step 5 (5 is the summation of steps 1-3). Small amounts of amide and diethyl halophosphate (the products of step 4) also were isolated.

$$2(\mathrm{EtO})_3\mathrm{P} + \mathrm{R'CONHX} \longrightarrow (\mathrm{EtO})_2\mathrm{P(O)H} + (\mathrm{EtO})_3\mathrm{PO} + \mathrm{R'CN} + \mathrm{EtX} \quad (5)$$

4. When one equivalent of triethyl phosphite was reacted with one equivalent of 7 in the presence of pyridine, the ratio of acetonitrile to ethyl bromide increased and pyridinium hydrobromide was isolated (cf Table 6). Pyridine, by acting as a scavenger for HBr formed in step 2, decreased the availability of HBr to react with triethyl phosphite (step 3).

5. When one equivalent of triethyl phosphite was reacted with one equivalent of 7 in the presence of one equivalent of HBr, the ratio of ethyl bromide to acetonitrile increased, indicating that HBr can compete with 7 for triethyl phosphite under the conditions of the reaction (cf Table 5).

The first step in the reaction sequence (step 1) for acyclic *N*-haloamides probably occurs through the imidoyl phosphonium halide as shown below. The formation of the imidoyl phosphonium halide is required by the isolation of *N*-methylacetimidoyl chloride from 12 and the isolation of the respective nitriles from 7, 8, 12, and 14.


(6)

$\mathrm{R} = \mathrm{CH_3}, \mathrm{C_2H_5}, n\text{-}\mathrm{C_4H_9}$

$\mathrm{R'} = \mathrm{CH_3}, \mathrm{C_6H_5}$

$\mathrm{R''} = \mathrm{H}, \mathrm{CH_3}$

$\mathrm{X} = \mathrm{Cl}, \mathrm{Br}$

The reaction between trimethyl phosphorothioite and 8 was reinvestigated in the light of above information. One equivalent of trimethyl phosphorothioite reacted with one equivalent of 8 to give approximately 0.3 equivalents each of benzamide, benzonitrile, and methyl chloride together with other products that were not identified. These products in large part explain the failure to isolate any dimethyl *N*-benzoylphosphoramidothioate from this reaction.

Table 6. EFFECT OF HBR ON THE REACTION BETWEEN *N*-BROMOACETAMIDE AND TRIETHYL PHOSPHITE IN BENZENE

| Moles of reagents | | | Moles of volatile products | |
|---|---|---|---|---|
| Haloamide | Phosphite | Other | EtBr | $CH_3CN$ |
| 1.0 | 1.0 | - | 0.37 | 0.48 |
| 1.0 | 1.0 | HBr - 1.0 | 0.74 | 0.08 |
| 1.0 | 1.0 | pyridine - 1.0 | 0.26 | 0.68 |

Conclusion - The reaction between trialkyl phosphites and acyclic primary halomides did not give the desired Arbuzov products but instead gave the variety of products indicated in Scheme I. Except for the cyclic haloamides, this procedure holds little promise for the synthesis of insecticidal O,S-dialkyl N-acylphosphoramidothioates.

## 3. Synthesis of Hydroxymethylcarbamates

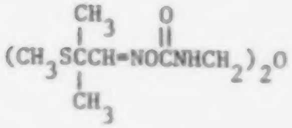
Introduction - During the course of our investigations on the synthesis and evaluation of N-derivatized methylcarbamate esters as selective insecticides, we discovered a convenient, high-yield method for the preparation of hydroxymethylcarbamates. These compounds are of considerable importance since they have often been found as metabolites in plants and animals treated with methylcarbamate esters.

Synthesis - The method described below is typical and is applicable to different methylcarbamte esters. The example given is for the preparation of 1-naphthyl hydroxymethylcarbamate (hydroxymethylcarbaryl).

A mixture of 5.6 g of 1-naphthyl methylcarbamate (0.03 mol), 40 ml of THF, 10 ml of water, 1.2g of paraformaldehyde (0.04 mol), and 1.0 ml of concentrated hydrochloric acid was stirred and heated gently to 50-60°C until a clear solution was obtained. The mixture was maintained at this temperature for 1 hr, then allowed to stand at room temperature for another hour. Dilution of the mixture with 100 ml of water produced an oil which was separated and the aqueous layer was extracted with three 50-ml portions of ether and the extracts were combined with the oil. The ether solution was washed with water, dried over anhydrous sodium sulfate, and the ether was removed to give an oil which solidified when concentrated under vacuum. Recrystallization from benzene and hexane gave 3.8g of 1-naphthyl hydroxymethylcarbamate (59% yield), mp 134-6°C. Pmr spectrum in $d_6$-dimethylsulfoxide with tetramethylsilane (TMS) as an internal standard showed a 2H doublet centered at $\delta$ 4.7 ($J$ = 7 Hz) for $NCH_2O$ protons and a 7H multiplet at $\delta$ 7.25-8.22 for the aromatic protons. Infrared spectrum in Nujol oil showed a band for NH, OH at 3300 $cm^{-1}$, and a carbonyl band at 1705 $cm^{-1}$.

Similar procedures were used for the synthesis of hydroxymethylcarbofuran and phenyl hydroxymethylcarbamate. In these cases the reaction mixture was neutralized with sodium carbonate at the end of the heating period and the THF solvent was removed under vacuum.

The above reaction was not applicable to the preparation of the hydroxymethyl derivative of aldicarb, an oxime carbamate. In this case the bis adduct of structure below was obtained.



$$(CH_3S\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{C}}CH{=}NO\overset{\overset{O}{\|}}{C}NHCH_2)_2O$$

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## I. INSECTICIDE CYCLIC NUCLEOTIDE INTERACTIONS

The fundamental discovery, that when certain hormones bind to their target cells there is an intracellular increase in cyclic nucleotides which then act as "second messengers" for these hormones, was originally made with the elucidation of the role of cyclic AMP (adenosine 3',5'-cyclic monophosphate) as the intracellular mediator of the hepatic glycogenolytic effect of epinephrine and glucagon.[1] Extensive subsequent studies of cyclic AMP have provided new insights into our understanding of the secretion of neurotransmitters, hormones, and other bioactive molecules, their actions at receptor sites, and the resultant regulatory mechanisms.[2] Most studies have been carried out on cyclic AMP and mammalian systems, and much less attention has been directed to cyclic GMP (guanosine 3',5'-cyclic monophosphate) or to invertebrate systems. Evidence continues to accumulate concerning the fundamental role of cyclic nucleotides in insects in morphogenesis and development,[3-6] nervous system activity,[7,8] flight muscle activity,[9,10] glandular secretion,[11,12] and control of cuticular tanning.[13,14] These studies have also primarily been on cyclic AMP, and surprising little attention has been directed to cyclic GMP even though it was first reported as early as 1969[15] that the cricket contained more cyclic GMP than cyclic AMP, the reverse of the situation reported for mammals. In 1971 several insect tissues were shown to have high cyclic GMP-dependent protein kinase activities,[16] and the authors concluded that "it is to be expected that further studies with arthropod material will be valuable for elucidating the respective roles of cyclic AMP and cyclic GMP in biological regulation." Yet arthropod tissues still essentially remain to be exploited in this regard.

Our interests in the cyclic nucleotides are multiple. There is the basic interest in characterization of these systems in insects. Our first studies[17,18] were investigations of the characteristics, previously unevaluated, of insect adenyl cyclase and phosphodiesterase, the enzymes responsible respectively for synthesizing and degrading cyclic AMP. Both enzymes were shown to be present in various tissues of the Madagascar cockroach, Gromphadorhina portentosa, and to have many of the same characteristics reported for the mammalian enzymes. We propose, as one future project, to investigate in the same manner the characteristics of guanyl cyclase and phosphodiesterase in insects. Secondly, we are interested in the basic involvement of cyclic nucleotides in insects as model systems for the further elucidation of neurotransmitter and hormone action at the target membrane, cellular, and systems levels. Thirdly, we are interested in the effects of drugs and foreign compounds on such systems. Cyclic nucleotide interactions are clearly involved in many biochemical phenomena in inter- and intra-cellular communication and differentiation and growth. Therefore, cyclic nucleotide actions should be relevant to the acute and chronic toxic and stress effects of foreign compounds in disrupting normal and effecting abnormal communication and regulation.

Several neurosecretory factors are proposed or known to be released from the insect CNS on insecticide poisoning.[19,20] Possible mechanisms for and consequences of increased Malpighian tubule secretion on insecticide poisoning in Rhodnius prolixus have been proposed involving the release of bound diuretic hormone by paralytic doses of a variety of insecticides, regardless of their primary modes of action on the CNS, and cyclic AMP as a second messenger.[21] Insecticide induced release of the hormone controlling integument elasticity in Rhodnius,[22] and the hyperglycaemic and adipokinetic hormones in Schistocerca gregaria[23] have also been reported.

In mammals, it has been reported that the incubation of rat renal cortices with DDT, chlordane, endrin, dieldrin, and heptachlor resulted in significant increases of cyclic AMP and that oral administration of these insecticides to rats resulted in an increase in endogenous cyclic AMP and the basal fluoride-stimulated activities of liver and kidney adenyl cyclase.[24,25] Recently, it has also been shown in in vitro and in vivo experiments with rats that dichlorvos, Dursban, and diazinon depressed endogenous corticosterone synthesis and blocked corticosteroidogenesis in response to ACTH and cyclic AMP stimulation of a suspension of adrenal cells.[26]

Studies of the possible interactions between insecticidal activity and the cyclic nucleotides as second messengers have potentially important implications to our more complete understanding of acute toxicity and particularly of chronic toxicity, which is essentially unresolved. Further, they may provide evidence for the actual systemic lesions which are responsible for the deaths of insects from insecticidal poisoning, also an area which is as yet not clearly elucidated, and of clues for the development of new toxicants with greater specificity.

Initially our studies[27] involved a survey of the direct effects of tepp, methyl paraoxon, DDT, dieldrin, aldicarb, dimetilan, rotenone, allethrin, and oxythioquinox on cockroach brain adenyl cyclase and phosphodiesterase in vitro. The results with cockroach brain adenyl cyclase in vitro appear in Table 1. In general they are highly variable and in no case do they show a classical inhibition curve. In all cases, except DDT, the high standard deviations and/or inconsistencies of action as inhibitor or activator with change in concentration lead to the conclusion that these compounds have essentially no direct effects on adenyl cyclase in vitro. With DDT, there is a relatively consistent inhibition at all concentrations, suggesting that DDT may be an inhibitor of adenyl cyclase. The absence of increased effect by DDT with increasing concentration may be due to solubility limitations.

The results with cockroach brain phosphodiesterase in vitro appear in Table 2. Dimetilan, rotenone, allethrin, and particularly methyl paraoxon show a general relationship of increasing inhibition with increasing concentration of insecticide, though effective concentrations are relatively high. On the other hand, DDT and particularly dieldrin

Table 1. DIRECT EFFECTS OF INSECTICIDES-ACARICIDES ON COCKROACH BRAIN ADENYL CYCLASE

Average protein concentration in each assay was 145 µg. Each value is the mean and standard deviation of three determinations.

| Compound | Percent effect on cockroach brain adenyl cyclase activity in comparison with controls | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $10^{-6}$ M | 5 x $10^{-6}$ M | $10^{-5}$ M | 5 x $10^{-5}$ M | $10^{-4}$ M | 5 x $10^{-4}$ M | $10^{-3}$ M | 5 x $10^{-3}$ M |
| Tepp | -- | -- | -- | -3.4±14.4 | +36.2±4.9 | +13.2±3.5 | -4.1±7.2 | +48.4±49.6 |
| Methyl paraoxon | -- | -- | -- | +16.5±25.7 | -7.7±26.6 | +0.9±10.9 | -57.8±1.9 | -29.3±41.0 |
| DDT | -- | -- | -- | -60.4±5.3 | -44.4±6.2 | -62.5±3.6 | -63.2±8.9 | -70. ±3.6 |
| Dieldrin | -- | -- | -28.0±1.1 | -48.9±5.8 | +24.4±12.4 | +16.0±48.2 | -44.1±8.6 | -- |
| Aldicarb | -- | -- | +16.4±33.6 | +14.1±22.0 | +57.0±43.6 | +28.6±5.0 | +2.4±1.2 | -- |
| Dimetilan | -- | -- | -- | -4.0±17.7 | -42.1±15.1 | -27.7±23.4 | -9.6±16.4 | -23.6±21.6 |
| Rotenone | -- | -- | +42.3±10.2 | +19.2±23.1 | +41.2±67.6 | -20.7±3.3 | -25.3±4.5 | -- |
| Allethrin | -- | -- | -18.2±47.2 | -32.2±16.7 | -10.2±36.3 | -55.0±11.7 | +15.2±7.4 | -- |
| Oxythioquinox | -8.1±0.9 | -26.0±2.2 | +27.8±35.6 | -25.4±9.9 | +2.4±38.2 | -- | -- | -- |

Table 2. DIRECT EFFECTS OF INSECTICIDES-ACARICIDES ON COCKROACH BRAIN PHOSPHODIESTERASE

Average protein concentration in each assay was 6.7 µg. Each value is the mean and standard deviation for three determinations.

| Compound | Percent effect on cockroach brain phosphodiesterase activity in comparison with controls | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $10^{-6}$ M | 5 x $10^{-6}$ M | $10^{-5}$ M | 5 x $10^{-5}$ M | $10^{-4}$ M | 5 x $10^{-4}$ M | $10^{-3}$ M | 5 x $10^{-3}$ M |
| Tepp | -- | -- | -- | +13.3±7.1 | -3.3±1.2 | +5.3±7.2 | -12.5±0.1 | -22.1±2.9 |
| Methyl paraoxon | -- | -- | -- | +1.2±8.0 | -28.9±1.8 | -34.7±8.1 | -80.6±2.1 | -86.5±3.5 |
| DDT | -- | -- | -- | +28.8±5.6 | +25.4±14.0 | +28.1±7.6 | +5.3±6.7 | +7.6±7.0 |
| Dieldrin | -- | -- | +0.5±6.0 | +45.0±2.7 | +54.1±4.4 | +75.7±6.0 | +64.0±5.3 | -- |
| Aldicarb | -- | -- | +8.0±7.0 | +10.5±3.0 | +15.2±5.6 | -5.4±7.7 | -14.2±1.8 | -- |
| Dimetilan | -- | -- | -- | -6.1±6.9 | -12.0±2.9 | -21.5±13.3 | -34.5±1.4 | -40.3±4.2 |
| Rotenone | -- | -- | -22.5±1.1 | -28.9±2.0 | -33.9±0.7 | -52.0±2.9 | -50.3±2.0 | -- |
| Allethrin | -- | -- | -3.4±6.5 | -2.5±7.1 | -32.0±32.7 | -37.0±9.8 | -36.7±7.2 | -- |
| Oxythioquinox | -89.7±2.9 | -87.3±1.7 | -85.8±1.7 | -83.8±4.2 | -81.7±1.3 | -- | -- | -- |

appear to be activators of phosphodiesterase. The inconsistencies of action as inhibitor or activator with increasing incubation concentrations suggest that tepp and aldicarb have no direct effect on phosphodiesterase *in vitro*. The most striking and potentially significant result of this exploratory survey was the observation that oxythioquinox is a potent inhibitor of cockroach brain phosphodiesterase, giving over 80% inhibition with an incubation concentration of 1 μM. The limited solubility of oxythioquinox in the buffer may explain similar levels of inhibition with increasing concentrations. In comparison, using identical assay techniques, 1000-fold greater concentrations (1 mM) of aminophylline and theophylline, the most widely used phosphodiesterase inhibitors in adenyl cyclase assays, inhibited 83.2% and 73.8%, respectively. This observation prompted us to obtain and evaluate the following series of quinoxalinedithiol derivatives (Fig. 1) as phosphodiesterase inhibitors.

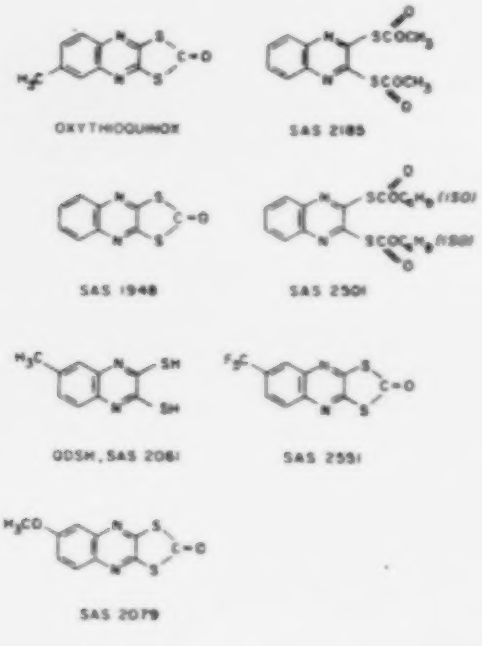

Figure 1. Chemical structures of quinoxalinedithiol derivatives.

The relative inhibition of cockroach brain phosphodiesterase by the various quinoxalinedithiol derivatives may be compared from their $I_{50}$ values given in Table 3.

Table 3. INHIBITION OF COCKROACH BRAIN PHOSPHODIESTERASE BY QUINOXALINEDITHIOL DERIVATIVES.

Assays contained an average of 6.48 μg protein. Inhibition concentrations ranging from $10^{-8}$ to $10^{-5}$ M were evaluated for each compound. Regression lines were calculated from at least three points per line and three replications per point.

| Compound | $I_{50}$ (M) |
|---|---|
| Oxythioquinox | $8.6 \times 10^{-7}$ |
| SAS 1948 | $3.2 \times 10^{-7}$ |
| SAS 2061 (QDSH) | $1.0 \times 10^{-7}$ |
| SAS 2079 | $1.6 \times 10^{-7}$ |
| SAS 2185 | $1.5 \times 10^{-6}$ |
| SAS 2501 | $4.2 \times 10^{-6}$ |
| SAS 2551 | $1.4 \times 10^{-7}$ |

A typical linear regression of log of inhibitor concentration versus mean percent inhibition is shown in Fig. 2 for SAS 2079.

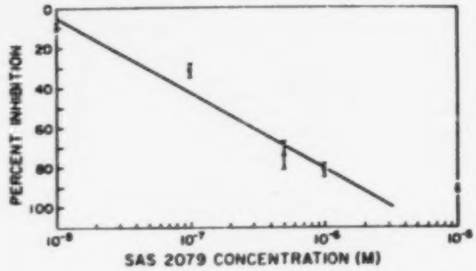

Figure 2. Inhibition of cockroach brain phosphodiesterase by SAS 2079. Each assay contained 6.3 μg protein. Each point is the mean of three determinations; brackets indicate standard deviation. Regression line is calculated from first four points.

Normally with increase in concentration of the quinoxaline inhibitors, inhibition reached limiting values of 70-90 percent, probably due to limits in solubility of the inhibitors in the incubation buffer at the higher concentrations. All of the quinoxalinedithiol derivatives examined were potent inhibtors of cockroach brain phosphodiesterase.

The inhibitory effects of caffeine, aminophylline, SQ 65,442, and the quinoxalinedithiol derivatives on cockroach brain, rat brain, and beef heart phosphodiesterases are compared in Table 4.

Table 4. THE EFFECTS OF METHYLXANTHINES, SQ 65,442, AND QUINOXALINEDITHIOL DERIVATIVES UPON THE ACTIVITY OF PHOSPHODIESTERASES FROM VARIOUS SOURCES.

Protein concentrations in the assays were as follows: cockroach: 6.53 μg; rat: 6.57 μg; beef heart: 5.52 μg. The incubation concentration of the methylxanthines was 1 mM, that of SQ 65,442 and quinoxalinedithiol derivatives was 1 μM. Each value is the mean and standard deviation of three determinations.

| Compound | Percent inhibition of phosphodiesterases from | | |
|---|---|---|---|
| | Cockroach brain | Rat brain | Beef heart |
| Caffeine | 45.6 2.5 | 56.0 3.7 | 60.3 1.7 |
| Aminophylline | 83.7 4.5 | 85.7 1.8 | 90.7 1.3 |
| SQ 65,442 | 48.5 3.7 | 66.9 1.6 | 13.0 3.5 |
| Oxythioquinox | 75.4 2.1 | 69.7 4.8 | 62.2 4.1 |
| SAS 1948 | 78.8 2.4 | 70.8 2.7 | 60.0 3.9 |
| SAS 2061 (QDSH) | 69.7 4.1 | 69.3 1.7 | 46.1 7.8 |
| SAS 2079 | 80.3 1.9 | 78.2 1.4 | 76.4 0.8 |
| SAS 2185 | 56.2 3.5 | 60.9 3.0 | 41.2 9.6 |
| SAS 2501 | 51.3 3.3 | 51.7 10.7 | 34.3 4.3 |
| SAS 2551 | 70.2 4.1 | 69.3 1.9 | 63.4 3.8 |

Generally the inhibitory activities of the quinoxalines and SQ 65,442, at 1000-fold less in concentration, were equal to or greater than caffeine and slightly less than aminophylline on all sources of phosphodiesterase studied. However, with the beef heart enzyme, the inhibitory activity of SQ 65,442 was markedly less than with the enzymes from cockroach and rat brains, and it was also somewhat lower with the beef heart enzyme for quinoxaline inhibitors. The methylxanthines were relatively equally inhibitory to the phosphodiesterases from all three sources. Overall the most potent inhibitor of phosphodiesterases from all three sources was SAS 2079. The inhibition of rat brain phosphodiesterase as a function of concentration of SAS 2079, SQ 65,442, and aminophylline is shown in Fig. 3.

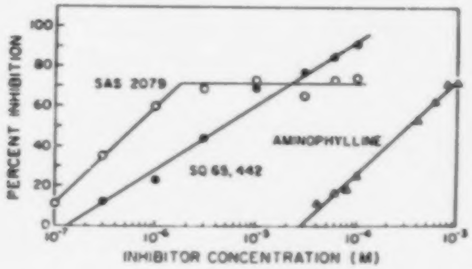

Figure 3. Inhibition of rat brain phosphodiesterase by SAS 2079, SQ 65,442, and aminophylline. Each assay contained 6.1 μg protein.

With SAS 2079, inhibition reached a limiting value of about 70% at concentrations above $10^{-6}$ M, whereas inhibition by SQ 65,442 and aminophylline increased linearly across the entire range of concentrations examined. Although the quinoxalinedithiol derivatives are generally more active inhibitors of phosphodiesterases at lower concentrations than are SQ 65,442 and the methylxanthines, it appears that solubility deficiencies limit their effectiveness, with inhibition reaching limiting values of about 70-90 percent as concentrations are increased.

Cheung[28] reported that bovine brain phosphodiesterase is inhibited by p-hydroxymercuribenzoate and the inhibition is reversible by 2-mercaptoethanol, indicating that the enzyme possesses sulfhydryl groups and that at least some of them are necessary for activity. This evidence, together with that of Carlson and DuBois[29] that oxythioquinox and QDSH are inhibitors of sulfhydryl enzymes of intermediate carbohydrate metabolism, suggests that the quinoxalinedithiol derivatives are also acting as sulfhydryl inhibitors of phosphodiesterase.

Oxythioquinox and other quinoxalinedithiol derivatives have been demonstrated to be potent *in vitro* inhibitors of phosphodiesterases from cockroach brain, rat brain, and beef heart, probably by acting as sulfhydryl inhibitors. However, any direct relationship of this activity to their mode of toxic action remains to be determined. On the basis of the broad distribution of phosphodiesterases in the animal kingdom, it seems unlikely that phosphodiesterase inhibition is a direct cause of their selective acaricidal activity. Although the quinoxalinedithiol inhibitors appear to be more active than any previously reported phosphodiesterase inhibitors,[30] their low water solubilities appear to cause inhibition to reach limiting values of about 70-90 percent as concentrations are increased in *in vitro* aqueous systems. Their evaluation as phosphodiesterase inhibitors to enhance the accumulation of cyclic AMP in adenyl cyclase assays of crude mitochondrial fraction of mouse brain did not demonstrate any significant advantage over the use of aminophylline, a broadly used standard inhibitor for this purpose.

Although direct *in vitro* inhibition of adenyl cyclase or phosphodiesterase was not generally shown by the insecticides in the above survey, it is possible that indirect rather than direct effects may be involved *in vivo* and that the insecticides may directly affect some other part of the system (release of a bioactive factor such as diuretic hormone, for example) which may then in turn affect cyclic nucleotide titers. Our subsequent work this year has been involved with examining this hypothesis by evaluating *in vivo* levels of both cyclic AMP and cyclic GMP in various cockroach tissues under normal and drug or insecticide challenged conditions.

The initial part of the work was directed toward familiarization and substantiation of bio-analytical methodology[31,32] for determining *in situ* levels of cyclic AMP and cyclic GMP in tissues of the Madagascar cockroach. Control levels have now been determined in brain, terminal abdominal ganglion, Malpighian tubules, rectum, and gastric caecae as summarized in the following table.

Table 5. NORMAL (CONTROL) LEVELS OF CYCLIC NUCLEOTIDES IN VARIOUS TISSUES OF THE MADAGASCAR COCKROACH

| Tissue | Cyclic nucleotides (pmole/mg protein) | |
|---|---|---|
| | Cyclic AMP | Cyclic GMP |
| Brain | 6.71 ± 0.48[a] | 17.43 ± 1.41 |
| Terminal abdominal ganglion | 2.74 ± 1.92 | 20.74 ± 15.93 |
| Malpighian tubules | 8.28 ± 1.23 | 29.06 ± 20.61 |
| Rectum | 3.10 ± 1.14 | 4.17 ± 2.22 |
| Gastric caecae | 2.42 ± 0.45 | 4.11 ± 2.47 |

[a] Average of three control determinations ± standard deviation.

The Malpighian tubules were found to have the highest control levels of both nucleotides. The CNS tissues, brain and terminal abdominal ganglion, were intermediate and the gut tissues, gastric caecae and rectum, were lowest. The high levels in Malpighian tubules, which are somewhat greater than those usually observed in mammalian tissues, suggest a probable extensive involvement of cyclic nucleotides in tubule function. Additionally, the higher levels of cyclic GMP than of cyclic AMP in all of the cockroach tissues is the reverse of that normally found in mammalian tissues and confirms the original observation from the cricket[15] noted above. These results also suggest that the Madagascar cockroach may have considerable potential for further studies on elucidating the respective roles of cyclic AMP and cyclic GMP in biological regulation. We anticipate undertaking an initial study in this regard to characterize guanyl cyclase and cyclic GMP phosphodiesterase, neither of which has been evaluated in insects, in comparison with our previous characterizations of the two cyclic AMP enzymes.

It has recently been reported[33] that both exposure to cold and administration of aminophylline (an inhibitor of phosphodiesterase) result in rapid increases in cyclic AMP *in vivo* in the rat adrenal medulla and adrenal cortex. In order to determine whether or not levels of cyclic AMP and cyclic GMP could be similarly affected in cockroach tissues by challenge with a known active drug, four male Madagascar cockroaches were injected with 200 pmole aminophylline/g body weight, sacrificed after 30 and 60 min, and the cyclic nucleotide levels determined in the pooled excised tissues: brain, terminal abdominal ganglion, Malpighian tubules, rectum, and gastric caecae, as summarized in the following table.

Table 6. LEVELS OF CYCLIC NUCLEOTIDES IN VARIOUS TISSUES OF THE MADAGASCAR COCKROACH 30 AND 60 MINUTES FOLLOWING INJECTION OF 200 pmole AMINOPHYLLINE/g BODY WEIGHT

| Tissue | Cyclic nucleotide (pmole/mg protein) | | |
|---|---|---|---|
| | Control | 30 min | 60 min |
| Cyclic AMP | | | |
| Brain | 6.18 | 8.81 | 7.06 |
| Terminal abdominal ganglion | 1.46 | 2.74 | 1.95 |
| Malpighian tubules | 9.17 | 15.95 | 14.28 |
| Rectum | 2.77 | 4.40 | 3.08 |
| Gastric caecae | 2.03 | 4.39 | 1.73 |
| Cyclic GMP | | | |
| Brain | 0 | 0 | 0 |
| Terminal abdominal ganglion | 0 | 0 | 6.81 |
| Malpighian tubules | 13.85 | 23.98 | 67.50 |
| Rectum | 1.74 | 10.83 | 4.10 |
| Gastric caecae | 2.13 | 3.41 | 2.83 |

The results show that inhibition of phosphodiesterase by injected aminophylline causes a marked increase in tissue levels of both cyclic nucleotides in 30 min, though the levels may then either increase or decrease by 60 min. They confirm a comparable response to aminophylline challenge in the cockroach to that found for rat adrenal medulla and adrenal cortex and suggest that this methodology might be appropriate for evaluating a challenge by insecticide poisoning, which then was our next experiment.

Sixteen male Madagascar cockroaches were injected with 40 μg of parathion in 4 μℓ of acetone, four cockroaches sacrificed at each of four different stages of poisoning symptoms, and the cyclic nucleotides determined in the pooled excised tissues: brain, terminal abdominal ganglion, Malpighian tubules, rectum, and gastric caecae. The four stages of poisoning symptoms are described in the following list. The times after injections shown are approximate averages, since the cockroaches were sacrificed by symptomology, not time.

| Stage | Time after injection (hr) | Description of symptomology |
|---|---|---|
| 1. Control | ~ 4.5 | No signs of effects from injection of 4 μℓ of acetone. |
| 2. Normal | ~ 0.5 | Antennae and palpi moving; normal exploration movements in cage. |
| 3. Active | ~ 1 | Rapid movements; spontaneous startle reaction and hissing behavior; abdomen shows tremors, flexing, and rotation; leg tremors. |
| 4. Incoordinated | ~ 2 | Hissing behavior and leg and body tremors accentuated; positional instability--insect falls on back with difficulty in righting, if righted again falls on back. |
| 5. Paralyzed | ~ 4 | On back; antennae in tetany; occasional leg and body tremors and flexing; abdomen bloated with secretion from anus. |

The cyclic nucleotide levels found in the various stages are shown in Table 7.

Table 7. LEVELS OF CYCLIC NUCLEOTIDES IN VARIOUS TISSUES OF THE MADAGASCAR COCKROACH AT VARIOUS STAGES OF SYMPTOMOLOGY FOLLOWING INJECTION OF 40 μg OF PARATHION PER COCKROACH

| Tissue | Cyclic nucleotides (pmole/mg) protein | | | | |
|---|---|---|---|---|---|
| | Control | Normal | Active | Incoordinated | Paralyzed |
| Cyclic AMP | | | | | |
| Brain | 7.13 | 15.22 | 10.88 | 10.48 | 5.04 |
| Terminal abdominal ganglion | 4.95 | 6.39 | 4.14 | 3.96 | 0 |
| Malpighian tubules | 6.88 | 10.81 | 19.03 | 9.34 | 11.49 |
| Rectum | 2.15 | 4.89 | 3.35 | 3.21 | 2.08 |
| Gastric caecae | 2.91 | 5.39 | 8.75 | 4.26 | 5.77 |
| Cyclic GMP | | | | | |
| Brain | 18.42 | 9.18 | 9.25 | 5.52 | 8.00 |
| Terminal abdominal ganglion | 9.47 | 7.36 | 7.53 | 8.08 | 0 |
| Malpighian tubules | 20.82 | 25.17 | 41.85 | 26.45 | 35.43 |
| Rectum | 4.69 | 5.40 | 5.84 | 3.99 | 6.26 |
| Gastric caecae | 6.88 | 4.48 | 29.34 | 108.82 | 98.78 |

Although the implications of these results are not yet understood, it is obvious that a number of interesting differential changes of cyclic nucleotide levels occurred. In the challenge with aminophylline there was an increase in level of both cyclic nucleotides in all tissues in 30 min. This was to be expected if aminophylline as a phosphodiesterase inhibitor protected the cyclic nucleotides being produced at a normal basal rate from normal degradative loss by enzymatic hydrolytic mechanisms. In the challenge with parathion, however, the changes in levels of cyclic nucleotides are much more specific and differential. Marked changes in levels occurred for both nucleotides for brain, Malpighian tubules and gastric caecae, but changes in levels were minimal for terminal abdominal ganglion and rectum. Differences in time sequence of changes in levels are also of potential significance. The maximum increase in cyclic AMP in the brain occurred in the normal stage, whereas in Malpighian tubules and gastric caecae it occurred in the active stage. The cyclic GMP levels decreased rather than increased in the brain, and in the Malpighian tubules they reached their maximum level in the active stage, whereas in the gastric caecae this occurred in the incoordinated stage. These differences suggest a time sequence of events in the different tissues and different involvement patterns for the two cyclic nucleotides. Obviously further investigation will be necessary for elucidation of these observations. They do, however, indicate a basic involvement of the cyclic nucleotides in the intoxication process. The elucidation of this involvement may hold keys to our more complete understanding of insecticidal intoxication and its consequences in both insects and mammals and particularly in relation to chronic toxicity.

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## J. INSECTICIDE PENETRATION AND ITS SPECIFICITY AS A RESISTANCE MECHANISM

Penetration of foreign compounds into the organism, together with partitioning and distribution factors within the organism, is probably the most inadequately elucidated aspect of toxicology. It is the generally accepted view that the integument or skin is directly penetrated by the foreign compound, and that once penetrated the foreign compound partitions into and is carried by the hemolymph or blood to be partitioned to the site of action in the tissues. In the past few years renewed interest in the penetration of contact insecticides into the insect body has been stimulated by a new hypothesis by P. Gerolt[1] that contact insecticides do not reach the site of action via the hemolymph, but follow a lateral route through the integument and tracheae to the cellular level. Although it is increasingly apparent that Gerolt's hypothesis is incorrect,[2,3] additional interest in penetration has been stimulated by the mounting evidence of the role of decreased penetration as a mechanism of insect resistance to insecticides.[2,4,5]

On the supposition that a comparison of insecticide absorption into susceptible and insecticide-resistant insects might provide a viable model system for studying factors of insecticide penetration, a survey was undertaken of the penetration of the insecticides used in resistance selection into susceptible and various house fly strains selected over the last twenty years. Penetration was determined by applying the insecticides to the cuticle, rinsing the treated insects with ethanol at various time intervals, and determining the amount of insecticide in the rinse by GLC analysis. Comparison of the penetration of 11 insecticides was made at the dosage levels (0.2 and 1.0 μg/fly) at 0, 1, 2, and 4 hours on two susceptible strains (NAIDM and WHO) and nine resistant strains [Super Pollard, MIPC (m-isopropylphenyl N-methylcarbamate), Ronnel, Ronnel II, DPEP (O-(2,4-dichlorophenyl) O-ethyl phosphoradmidothioate), Fenthion, Parathion, Dimethoate, and Chlorthion].

A plot of μg absorbed/female fly versus time for ronnel, the most completely absorbed of the compounds studied, is shown in Figure 1. This plot is typical of most of the insecticides and strains studied. The greatest differences in absorption between strains were at 1 hour, relatively equal amounts being absorbed by 4 hours. The initial rate of absorption was greater for the susceptible than for the resistant strains. Greater actual amounts were absorbed at the higher than the lower dosage.

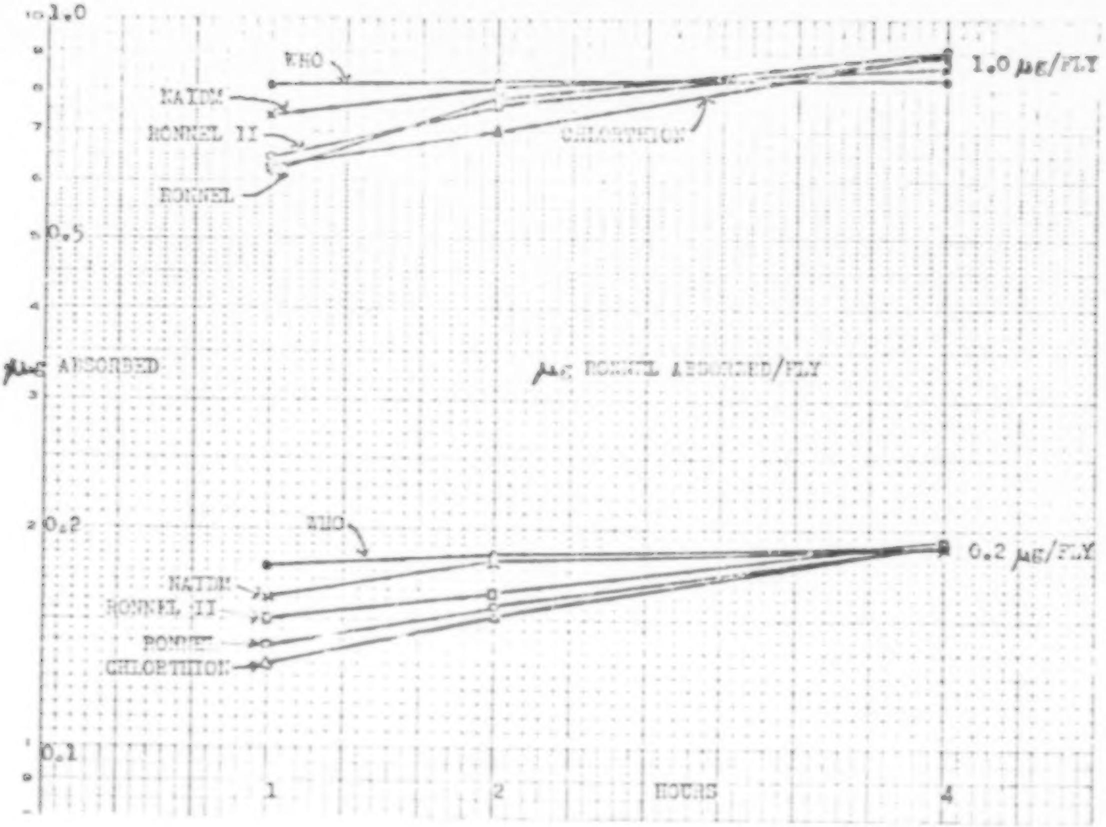


Figure 1. Absorption of ronnel by various house fly strains.

A similar plot for dimethoate (also typical of dieldrin, DDT, and fenoxon) is shown in Figure 2. Note that here less of the higher dosage is absorbed initially than of the lower dosage by the resistant strains. Such an inverse relationship has not been reported previously, and this is one observation of this initial survey which merits further verification and elucidation.

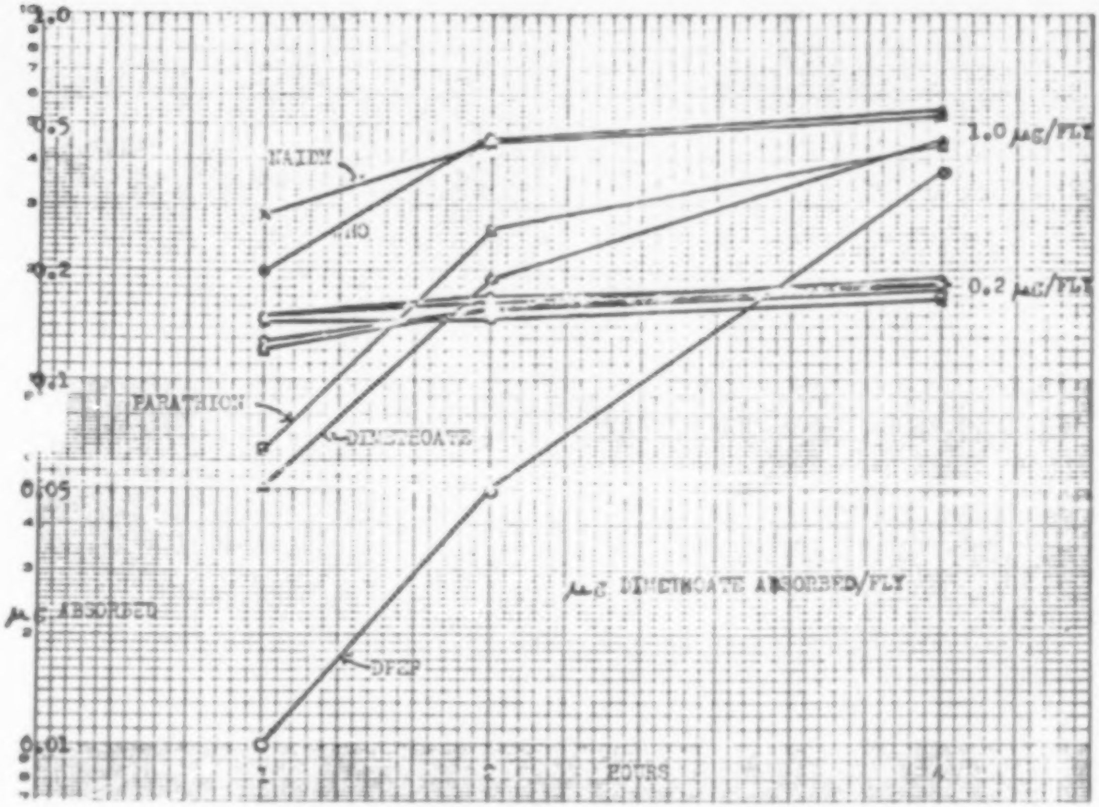
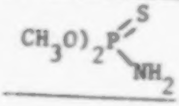


Figure 2. Absorption of dimethoate by various house fly strains.

The initial survey disclosed that reduced penetration is primarily operative within the first few hours following application, for by four hours penetration of a given insecticide is nearly equal in all strains. A summary of the absorption data one hour after application, at which maximum differences were observed, is shown in Table 1.

Table 1. PERCENT INSECTICIDE ABSORBED[a] IN THE HOUSE FLY ONE HOUR AFTER TOPICAL APPLICATION OF 0.2 OR 1.0 μg/FEMALE FLY.

House fly strains selected for resistance with each insecticide underlined.



$CH_3O)_2P(=S)NH_2$

| Strain | 0.2 | 1.0 |
|---|---|---|
| G.P.[b] | 100 | 100 |
| P.C.[c] | 72 | 80 |
| NAIDM | 95 | 99 |
| WHO | | 99 |
| S. Pollard | 95±1[d] | 99±1[d] |
| MIPC | | 99 |
| Ronnel | 95 | 99 |
| Ronnel II | | 99 |
| DPEP | | 99 |
| Parathion | | 99 |
| Fenthion | | 99 |
| Chlorthion | | 99 |
| Dimethoate | | 99 |

Ronnel

| Strain | 0.2 | 1.0 |
|---|---|---|
| G.P. | 62 | 15 |
| P.C. | 38 | 35 |
| WHO | 89 | 81 |
| NAIDM | 81 | 74 |
| Parathion | 77 | 67 |
| Fenthion | 75 | 67 |
| MIPC | 74 | 67 |
| Dimethoate | 71 | 67 |
| Ronnel II | 75 | 64 |
| Chlorthion | 65 | 63 |
| Ronnel | 70 | 62 |
| S. Pollard | 63±2 | 57±11 |
| DPEP | 79 | 42±18 |

DPEP

| Strain | 0.2 | 1.0 |
|---|---|---|
| G.P. | 18 | 5 |
| P.C. | 46 | 10 |
| WHO | 80 | 62 |
| NAIDM | 78 | 61 |
| S. Pollard | 66±4 | 52±4 |
| MIPC | 66 | 48 |
| Fenthion | 74 | 43 |
| Ronnel | 59 | 43 |
| Ronnel II | 57 | 39 |
| Parathion | 72 | 37 |
| Chlorthion | 60 | 36 |
| Dimethoate | 66 | 34±10 |
| DPEP | 60 | 26±6 |

Parathion

| Strain | 0.2 | 1.0 |
|---|---|---|
| G.P. | 24 | 0 |
| P.C. | 20 | 5 |
| NAIDM | 81 | 61 |
| WHO | 81 | 57 |
| MIPC | 70 | 46 |
| Ronnel II | 74 | 36 |
| Ronnel | 70 | 35 |
| Chlorthion | 58 | 32 |
| DPEP | 76 | 30 |
| Fenthion | 74 | 30 |
| Parathion | 67 | 28 |
| S. Pollard | 55±12 | 26±11 |
| Dimethoate | 58 | 25±7 |

Table 1 (continued). PERCENT INSECTICIDE ABSORBED IN THE HOUSE FLY

| | Fenthion | |
|---|---|---|
| Strain | 0.2 | 1.0 |
| G.P. | 22 | 0 |
| P.C. | 20 | 0 |
| NAIDM | 79 | 61 |
| WHO | 79 | 58 |
| MIPC | 58 | 34 |
| Fenthion | 59 | 33 |
| Ronnel II | 56 | 33 |
| S. Pollard | 46±13 | 33±7 |
| DPEP | 59 | 32 |
| Parathion | 56 | 29 |
| Chlorthion | 41 | 27 |
| Dimethoate | 52 | 23 |
| Ronnel | 56 | 22 |

| | Monitor | |
|---|---|---|
| | 0.2 | 1.0 |
| G.P. | 46 | 5 |
| P.C. | 36 | 30 |
| WHO | 84 | 60 |
| MIPC | 80 | 58 |
| DPEP | 58 | 52 |
| NAIDM | 74 | 47 |
| S. Pollard | 84±1 | 46±2 |
| Ronnel II | 66 | 46 |
| Fenthion | 60 | - |
| Dimethoate | 60 | - |
| Ronnel | 44 | 46 |
| Parathion | 66 | 40 |
| Chlorthion | 44 | 36 |

| | Chlorthion | |
|---|---|---|
| Strain | 0.2 | 1.0 |
| G.P. | 0 | 0 |
| P.C. | 16 | 0 |
| NAIDM | 84 | 58 |
| WHO | 87 | 57 |
| MIPC | 77 | 32 |
| Fenthion | 62 | 29 |
| S. Pollard | 64±5 | 24±4 |
| Parathion | 69 | 23 |
| Ronnel II | 64 | 23 |
| Ronnel | 69 | 19 |
| Dimethoate | 68 | 19 |
| Chlorthion | 71 | 18 |
| DPEP | 72 | 16±13 |

| | Dieldrin | |
|---|---|---|
| Strain | 0.2 | 1.0 |
| G. P. | 24 | 0 |
| P.C. | 52 | 0 |
| WHO | 85 | 54 |
| NAIDM | 71 | 52 |
| MIPC | 61 | 30 |
| DPEP | 70 | 26 |
| S. Pollard | 50±5 | 25±4 |
| Ronnel | 68 | 21 |
| Dimethoate | 72 | 19±8 |
| Fenthion | 52 | 14 |
| Chlorthion | 54 | 13 |
| Ronnel II | 60 | 12 |
| Parathion | 63 | 5 |

Table 1 (continued). PERCENT INSECTICIDE ABSORBED IN THE HOUSE FLY

| Fenoxon<br>Strain | 0.2 | 1.0 |
|---|---|---|
| G.P. | 12 | 0 |
| P.C. | 12 | 15 |
| WHO | 68 | 44 |
| NAIDM | 73 | 42 |
| S. Pollard | 76±3 | 36 |
| Ronnel II | 70 | 34 |
| Ronnel | 64 | 28 |
| MIPC | 67 | 18 |
| Parathion | 57 | 15 |
| Dimethoate | 66 | 13 |
| DPEP | 70 | 12 |
| Chlorthion | 60 | 8 |
| Fenthion | 47 | 3 |

| DDT<br>Strain | 0.2 | 1.0 |
|---|---|---|
| G.P. | 44 | 0 |
| P.C. | 42 | 10 |
| WHO | 57 | 39 |
| NAIDM | 52 | 29 |
| MIPC | 51 | 22 |
| Fenthion | 40 | 13 |
| Ronnel | 48 | 12 |
| DPEP | 47 | 12±4 |
| Chlorthion | 44 | 12 |
| Parathion | 57 | 10±4 |
| Dimethoate | 48 | 9 |
| S. Pollard | 34±6 | 9±7 |
| Ronnel II | 36 | 6 |

| Dimethoate<br>Strain | 0.2 | 1.0 |
|---|---|---|
| G.P. | 4 | 0 |
| P.C. | 38 | 25 |
| NAIDM | 72 | 28±1 |
| MIPC | 64 | 27 |
| Ronnel | 71 | 25 |
| WHO | 74 | 20±8 |
| Ronnel II | 60 | 20 |
| S. Pollard | 64±10 | 17±8 |
| Fenthion | 72 | 14±6 |
| Chlorthion | 68 | 8±12 |
| Parathion | 60 | 6±2 |
| Dimethoate | 74 | 5±1 |
| DPEP | 63 | 2±8 |

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[a] Lost from cuticular surface.

[b] G.P. = Glass pipet treated with insecticide in ethanol (0.5 μl) containing extractable cuticular components of 25 house flies/ml.

[c] P.C. = Empty NAIDM house fly puparial case treated with insecticide in ethanol (0.5 μl).

[d] Average ± S.D.

These data disclose two striking results for further study. As noted above (Fig. 2), with dimethoate, DDT, fenoxon, and dieldrin, one or more of the most penetration-resistant strains absorb less actual insecticide for the higher than the lower dosage at 1 hour, whereas the reverse and expected pattern is shown at later times and by the other strains and for other insecticides. Delayed penetration has previously been characterized as non-specific and has generally been considered to be related to physical properties such as solubility. A much more specific pattern appears to emerge from this initial survey which is suggestive of structure-activity rather than physical property relationships. This is suggested by the changing pattern of relative absorption by the different strains for the various insecticides and particularly by the fact that the strain selected with a particular insecticide is among the least absorptive of that insecticide as summarized in Table 2 showing the rank order of house fly strains in decreasing order of absorption of insecticides at 1.0 μg/fly dose.

Table 2. RANK ORDER OF HOUSE FLY STRAINS IN DECREASING ORDER OF ABSORPTION OF INSECTICIDES AT 1.0 μg/FLY DOSE (FROM TABLE 1)

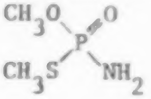
| Insecticide | Rank order of strains (1 = most absorptive) | | | | | |
|---|---|---|---|---|---|---|
| | WHO | NAIDM | MIPC | Fenthion | S. Pollard | Ronnel II |
| Ronnel | 1 | 2 | 5 | 4 | 10 | 7 |
| DPEP | 1 | 2 | 4 | 5 | 3 | 7 |
| Parathion | 2 | 1 | 3 | 8 | 10 | 4 |
| Fenthion | 2 | 1 | 3 | 4[a] | 6 | 5 |
| Monitor | 1 | 4 | 2 | 7 | 5 | 6 |
| Chlorthion | 2 | 1 | 3 | 4 | 5 | 7 |
| Dieldrin | 1 | 2 | 3 | 8 | 5 | 10 |
| Fenoxon | 1 | 2 | 6 | 11 | 3 | 4 |
| DDT | 1 | 2 | 3 | 4 | 10 | 11 |
| Dimethoate | 4 | 1 | 2 | 7 | 6 | 5 |
| Ave. Rank | 1.6 | 1.8 | 3.4 | 6.2 | 6.3 | 6.6 |

| | Ronnel | Parathion | DPEP | Chlorthion | Dimethoate |
|---|---|---|---|---|---|
| Ronnel | 9 | 3 | 11 | 8 | 6 |
| DPEP | 6 | 8 | 11 | 9 | 10 |
| Parathion | 5 | 9 | 7 | 6 | 11 |
| Fenthion | 11 | 8 | 7 | 9 | 10 |
| Monitor | 9 | 10 | 3 | 11 | 8 |
| Chlorthion | 8 | 6 | 11 | 10 | 9 |
| Dieldrin | 6 | 11 | 4 | 9 | 7 |
| Fenoxon | 5 | 7 | 9 | 10 | 8 |
| DDT | 5 | 8 | 6 | 7 | 9 |
| Dimethoate | 3 | 9 | 11 | 8 | 10 |
| Ave. Rank | 6.7 | 7.9 | 8.0 | 8.7 | 8.8 |

[a] Rank order of strain for insecticide it was selected with underlined.

This is also supported by the fact that no direct relationship between water solubility or partitioning behavior of the insecticides and their degrees of absorption in the susceptible NAIDM strain was found, as shown in Table 3.

Table 3. WATER SOLUBILITY, PARTITIONING COEFFICIENTS, AND CUTICULAR ABSORPTION OF VARIOUS INSECTICIDES IN THE NAIDM STRAIN OF THE HOUSE FLY

| Insecticide | Water solubility | Partitioning coefficient[6] | % of applied dose absorbed | | | |
|---|---|---|---|---|---|---|
| | | Isooctane: | 1 hour | | 4 hours | |
| | PPM | 80% acetone | 0.2 µg | 1.0 µg | 0.2 µg | 1.0 µg |
| DDT | 0.0034 | 0.93 | 52 | 29 | 80 | 59 |
| Dieldrin | 0.25 | 0.88 | 71 | 52 | 91 | 74 |
| DPEP | 5 | | 78 | 61 | 95 | 80 |
| Parathion | 24 | 0.76 | 81 | 61 | 93 | 79 |
| Chlorthion | 40 | 0.56 | 84 | 58 | 99+ | 83 |
| Ronnel | 44 | | 81 | 74 | 95 | 86 |
| Fenthion | 55 | | 79 | 61 | 94 | 83 |
| Fenoxon | 5500 | | 73 | 42 | 94 | 68 |
| Dimethoate | ca. 20,000 | | 72 | 28 | 84 | 52 |
|  | soluble | | 74 | 47 | 88 | 67 |

A third piece of evidence that the penetration-delaying mechanism (and thus perhaps the penetration mechanism itself) may be more specific than non-specific is provided by a study of the absorption of and resistance to tributyltin chloride (TBTCl) in the house fly strains.

TBTCl has been utilized as a diagnostic toxicant in identifying the absorption delaying mechanisms for resistance controlled by gene(s) on chromosome III in the house fly.[4,5] The resistant house fly strains used in these studies show relative resistances of 10- to 32-fold to TBTCl when compared at the $LD_{50}$ level to the susceptible NAIDM strain (see Table 4). With the thought that evaluation of absorption of TBTCl in comparison to resistance to TBTCl in the house fly strains might assist in interpreting the data on absorption patterns of other insecticides in the strains, a GLC method for TBTCl was worked out. Conditions were as follows: 2 ft 4 mm ID glass U-column packed with 5% OV101 on Gas Chrom Q 80/100 mesh; temperatures: column oven 158°, flash heater 290°, detector 250°; gas flows (ml/min): H 33, He 40, air 330; FID detector, minimum quantitatively detectable TBTCl 250 ng. When absorption of TBTCl into the various strains was determined, no relationship of absorption to resistance level was found as shown in Table 4. There is also no relationship between resistance ratio as an indicator of level of delayed absorption as a resistance mechanism and the average rank order of absorption of the various strains as shown in Table 2. Although these results were thus of little help in interpreting the other absorption data, they raise a number of questions for further investigations on the role and mechanisms of TBTCl resistance.

Table 4. TOXICITY AND RESISTANCE RATIOS FOR TRIBUTYLIN CHLORIDE IN THE HOUSE FLY IN COMPARISON WITH PER CENT ABSORPTION[a] 1 HOUR AFTER TOPICAL APPLICATION OF 10 µg/FEMALE FLY

| Strain | 24-hour $LD_{50}$ µg/female fly | Resistance ratio $\frac{LD_{50}\text{ strain X}}{LD_{50}\text{ NAIDM}}$ | % TBTCl absorbed (lost) in 1 hour |
|---|---|---|---|
| G.P.[b] | | | 24 |
| P.C.[c] | | | 33 |
| NAIDM | 0.59 | 1 | 51 |
| WHO | 0.62 | 1 | 62 |
| S. Pollard | 5.9 | 10 | 64 |
| Dimethoate | 8.4 | 14 | 64 |
| Parathion | 8.4 | 14 | 59 |
| MIPC | 9.1 | 15 | 54 |
| Chlorthion | 10.0 | 17 | 60 |
| Ronnel | 12.3 | 21 | 60 |
| DPEP | 18.0 | 30 | 68 |
| Fenthion | 19.0 | 32 | 58 |

[a] Loss from cuticular surface.

[b] G.P. = Glass pipet treated with insecticide in ethanol (0.5 µl) containing extractable cuticular components of 25 house flies/ml.

[c] P.C. = Empty NAIDM house fly puparial case treated with insecticide in ethanol (0.5 µl).

This initial survey suggested a number of potential entries for further investigation of the fundamental aspects of insecticide penetration and its interrelationships with metabolism, toxicity, and resistance. But before attempting more critical and fundamental extensions of the work, our attention was directed toward further elucidation and interpretation of these initial studies.

For example, there was the question of significance of differences between the individual absorption figures. It was not difficult to accept the large differences between the most and least absorbing strains as being significant since these differences were normally 20% or more, but was a difference of 5% or even 10% significant? Since the logistics of making all determinations in sufficient replications to assess variability was insurmountable, the expedient of making replicate (4) determinations of absorption for all insecticides with a single strain and for a few selected insecticides with other strains was resorted to. A summary of the absorption data, including the replicate averages and their standard deviations, 1 hour after application is found in Table 1. The data for 1 hour are utilized for comparison since the differences in absorption between strains were greatest at this time. Greater differences are also apparent at the higher than lower dosage. These observations suggest that the initial rate of absorption is the most critical difference factor and that the delayed absorption mechanism is more operative at the higher than lower dose. Thus, further studies of absorption rates within the first hour after application of insecticide and the effects of dosage level merit further consideration. The replicate averages and their standard deviations give some confidence that there are significant differences in absorption between the two susceptible strains and the nine resistant strains, but the significance of differences between the resistant strains is much less clear, although in a number of cases there appear at least to be significant differences between the most and least absorbing of the resistant strains.

However, the question of non-specificity or specificity of absorption mechanisms and of delayed absorption as a resistance mechanism remains unresolved since many of the differences in absorption between the resistant strains do not appear to be significant. If the mechanisms were non-specific and related only to physical properties such as solubility or partitioning properties, one would expect that the strains would fall into a common rank order for all of the insecticides in relation to the degree of development of the delayed penetration resistance mechanism. It seems reasonably apparent from the rank orders of absorption presented in Table 2 that the two susceptible strains quite uniformly absorb the most insecticide and that the MIPC resistant strain does the same among the resistant strains. There is considerable non-uniformity of rank among the remaining resistant strains, however.

Perhaps most striking is the observation that the rank order of a strain for the insecticide it was selected with is normally very high, which suggests that there may be some specificity for delayed absorption of the selecting insecticide. Although this order appears to be something more than random, it is apparent that additional studies will be required to assess the question of non-specificity of absorption as related to physical properties such as solubility or partitioning or of specificity of absorption as related to chemical structure-activity properties such as binding to cuticular components or carrier molecules.

In the initial survey, there did not appear to be any direct relationship between solubility or partitioning properties and absorption. Since losses might occur in the methods used by volatilization as well as absorption, it was decided to investigate relative volatility of the various insecticides so that losses from this source might be corrected. It was soon observed that a number of the insecticides volatilized from a glass surface, but this did not seem to be a reasonable comparison to the house fly cuticle, since cuticular components might be expected to have retarding effects on volatility. Therefore, measurements were made of losses from glass (disposable glass pipets) by insecticides applied in ethanol (0.5 μl) containing the extractable cuticular components of 25 house flies/ml. The cuticular components had a deterring effect on volatilization. Measurements of losses were also made by applying the insecticides in ethanol to empty house fly puparial cases (NAIDM) with the reasoning that these might closely approximate the adult cuticular surface, but that the effects of absorption (the insect pupa is a notably insecticide-tolerant stage usually attributed to poor absorption) and the metabolism might be bypassed. The data for these measurements are also shown in Table 1 under the headings G.P. (glass pipet) and P.C. (puparial case). It is apparent that they do not provide a completely adequate pseudo-fly for measuring volatility corrections, but primarily serve to point out the complicated interactions of the many factors involved--the effects of dose on volatility and absorption, of cuticular components on volatility and extractability, of partitioning from the cuticle to other tissues, of metabolism, of excretion, etc. If either of the two methods were to be used in further studies, it appears that the glass pipet method might be most representative of volatility losses _per se_. They also point out that further studies on the relationship of absorption to solubility and partitioning properties must contain an estimate of losses due to volatility.

## References

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## K. GAS CHROMATOGRAPHY OF INSECTICIDES ON MODIFIED SUPPORTS

The direct gas chromatography of most carbamate and many phosphate insecticides has presented difficulties because of on-column pyrolysis or retention on reactive sites. Thus we have been activity investigating GLC methods to improve our capabilities in this regard. Recently, the development of a novel gas chromatographic phase, obtained by heat treatment and exhaustive methanol extraction of a 6% Carbowax 20M packing was reported.[1] This new phase allowed fast, efficient, and selective separations of several types of polar compounds with little indication of decomposition of labile compounds. The new methodology showed characteristics of the more desirable features of both gas-solid and gas-liquid chromatography--low bleed, short retention times, high selectivity, and excellent peak symmetry and resolution of highly polar compounds. Further studies have demonstrated that the methodology is not limited to Carbowax 20M as a coating phase.[2,3] The application of the modified Carbowax 20M support to the direct gas chromatography of carbamate insecticides has also been demonstrated,[4] but this novel methodology has not been extended to other pesticides or the modification of other support phases for the gas chromatography of pesticides.

We have now investigated and extended the use of this new support methodology to the gas chromatography of a number of carbamates and phosphates, which we have previously been unsuccessful in chromatographing, as well as in chromatographing a number of chlorinated hydrocarbon insecticides. Our initial studies were with a modified Carbowax 20M column. The packing was prepared by coating HCl-washed Chromosorb-W 80/100 with 6% Carbowax 20M. The coated support was conditioned overnight at 280° and then exhaustively extracted with methanol, producing a solid support presumably surface-modified with a monomolecular layer of Carbowax 20M. The GLC conditions utilizing a 6 ft 2 mm I.D. glass U-column were as follows: carbamates and phosphates: $Rb_2SO_4$ AFID, H 23 ml/min, He 30 ml/min, air 234 ml/min, and oven temperatures 135-185°; chlorinated hydrocarbons: EC detector, Argon (95)-methane (5) 30 ml/min, and oven temperatures 165-225°C. This new methodology appears most promising in permitting us to utilize GLC analysis for a number of previously quantitatively unchromatographable compounds in our studies on insecticide absorption, as shown in Table 1. Further, the modified Carbowax 20M column almost appears to be a universal column for multi-classes of insecticides. A limiting characteristic of this column, however, was the failure to achieve separation of phosphorothionates and their oxygen analogs, which would limit its applicability to many types of uses.

Table 1. RETENTION TIME IN MINUTES FOR VARIOUS CARBAMATE, PHOSPHATE, PHOSPHOROTHIONATE, AND CHLORINATED HYDROCARBON INSECTICIDES ON A CARBOWAX 20M-SURFACE-MODIFIED GLC COLUMN

| Insecticide | Retention time in min at following temperatures | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 135° | 145° | 155° | 165° | 170° | 185° | 205° | 225° |
| Temik | 4.4 | 2.8 | | | | | | |
| Methomyl | 10.5 | 6.4 | 3.9 | | | | | |
| MIPC | | | 2.4 | | 1.5 | | | |
| Propoxur | | | 3.0 | | 2.0 | | | |
| Zectran | | | 6.7 | | 4.4 | 1.8 | | |
| Pyrolan | | | 10.2 | | 6.8 | 2.6 | | |
| Carbaryl | | | | | 12.5 | 3.5 | | |
| Monitor | | | 1.7 | | 1.5 | | | |
| Dyfonate | | | 2.3 | | 2.0 | | | |
| Orthene | | | 4.6 | | 3.0 | | | |
| Dimethoxon | | | | | 4.5 | | | |
| Dasanit | | | | | 5.0 | | | |
| Fenoxon | | | | | 7.0 | | | |
| Paraoxon | | | | | 7.5 | 3.0 | | |
| Chloroxon | | | | | 11.0 | 4.0 | | |
| Ciodrin | | | | | 15.0 | 6.4 | | |
| Phenkapton | | | | | | 12.0 | | |
| Lindane | | | | 1.2 | | 0.6 | | |
| Aldrin | | | | 1.5 | | 0.8 | 0.4 | |
| Dieldrin | | | | 4.6 | | 2.2 | 1.1 | |
| Kepone | | | | | | | 2.9,6.0 | 1.3,5.6 |
| Mirex | | | | | | | 2.4 | 1.3 |
| DDT | | | | | | 5.0 | 2.2 | 1.0 |

Therefore, we have investigated modified supports prepared with a number of other gas chromatography phases: SE 30, stabilized DEGS, OV 101, QF 1, Dexil 300, and Silar 9CP. Modified supports prepared with the first five phases above showed variable and limited usefulness in comparison with the modified Carbowax 20M column. However, our most recent trials with a modified Silar 9CP column have shown excellent chromatography and separations of phosphorothionates and their oxygen analogs as shown in Table 2. This column has provided greater separation of phosphorothionates and their oxygen analogs than any column we have ever examined. Further, it is the first column on which we have successfully chromatographed compound 8579. This study is being continued with the preparation of additional modified supports and the examination of chromatography of additional pesticides.

Table 2. RETENTION TIME IN MINUTES FOR VARIOUS PHOSPHOROTHIONATE AND PHOSPHATE INSECTICIDES ON A SILAR 9CP-SURFACE-MODIFIED GLC COLUMN.

| Insecticide | Retention time in min at following temperatures | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 140° | 155° | 170° | 185° | 200° | 215° | 230° | 245° | 260° |
| Bidrin | 1.6 | 1.0 | 0.8 | 0.6 | | | | | |
| Dyfonate | 3.2 | 1.7 | 1.1 | | | | | | |
| Dioxathion | | | 1.4 | 0.9 | 0.7 | | | | |
| Fenthion | | | 2.0 | 1.2 | 1.0 | | | | |
| Fenoxon | | | | 5.8 | 3.3 | 1.8 | | | |
| 8125[a] | | | 2.5 | 1.4 | 1.0 | | | | |
| 8579[b] | | | | 2.1 | 1.2 | 0.9 | | | |
| Chlorthion | | | 2.8 | 1.6 | 1.1 | | | | |
| Chloroxon | | | | 4.5 | 2.5 | 1.4 | | | |
| Methamidphos | | | 2.8 | 1.7 | 1.2 | | | | |
| Acephate | | | | | 2.2 | 1.3 | 0.9 | | |
| Parathion | | | | 2.0 | 1.3 | 0.9 | | | |
| Paraoxon | | | | 6.8 | 3.7 | 2.0 | | | |
| Dimethoate | | | | 2.8 | 1.7 | 1.1 | | | |
| Dimethoxon | | | | | 3.9 | 2.1 | 1.3 | | |
| Carbophenothion | | | | 2.8 | 1.7 | 1.1 | | | |
| Rueline | | | | | | | 2.6 | 1.9 | 1.1 |
| Dasanit | | | | | | | 3.7 | 2.4 | 1.4 |

[a] O-(2,4-dichlorophenyl) O-ethyl phosphoramidothioate

[b] 2,4-dichlorophenyl ethyl phosphoramidate

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## L. NEUROPHYSIOLOGICAL STUDIES ON THE MODE OF ACTION OF INSECTICIDES

Studies on the action of insecticides on insects were carried out at two levels. First, the poisoning process of known insecticides was studied by recording motor units from walking and flying house flies before, during, and after insecticide treatment. Over the course of the grant period this preparation became more sophisticated as the central nervous system was isolated. Second, the actions of neurally active compounds were measured at discrete tissues or subcellular elements. This usually involved the insect neuromuscular junction as a model system. Recently, the second half of the project has been expanded in two ways: To do a structure-activity study of antagonists of inhibitory synaptic transmission and to evaluate neurally active compounds for insect and mammalian convulsant properties. These latter approaches were deliberately designed to gain more information about the selective toxicity of newer types of chemicals.

### 1. Synaptic Preparations

Visceral neuromuscular junction - Many, but not all, visceral muscles in insects are innervated by neurosecretory axons. The neurosecretory junctions within the heart muscle of the American cockroach, *Periplaneta americana*, show ultrastructural and electrophysiological evidence of chemically transmitting synapses and cytochemical evidence for the presence of monoamines. Electron microscopy of nerve terminals shows that synaptic vesicles may be formed directly from electron-dense "neurosecretory" granules.

The alary muscles of the cockroach, *P. americana*, are striated with an A-band of 3.0 to 3.5 μm long. Each muscle fiber was 10 to 12 μm in diameter, and Z-lines appeared as small discrete units staggered throughout the sarcoplasm. Mitochondria were conspicuously located near the Z-line areas and were absent from the middle portion of the sarcomere. A transverse membrane system was present which formed dyad structures with a relatively sparse sarcoplasmic reticulum. Cockroach alary muscles were innervated by axons containing electron-dense granules of near 100 nm in diameter. These are thought to be typical of "neurosecretory" axons based on their ultrastructural appearance.

The hyperneural muscle of *P. americana* is striated with an A-band at least 2 μm long. Z-bands were discrete units, but arranged with some order in the myoplasm. The sarcoplasmic reticulum was reduced and 1 thick was surrounded by 10 to 12 thin myofilaments. The muscle is innervated from the median nerves by axons containing electron-dense granules which may be opaque near the neuromuscular junction amid numerous synaptic vesicles. Depolarizing intracellular current injection produces an ohmic voltage response of the membrane potential, and neurally evoked contraction is effected by summated excitatory postsynaptic potentials. All contractal activity ceases when the innervation is removed. The muscle appears to be electrically inexcitable and to be under obligatory control of central motor units. By virtue of attachments along the ventral nerve cord in the abdomen, the hyperneural muscle, when activated, moves the nerve cord unidirectionally. In effect, the hyperneural muscle is called upon by the central nervous system to move the ventral nerve cord presumably into a greater mix with the hemolymph in response to as yet unknown stimuli.

A new transducer was designed and built to measure the extremely fine movements of the hyperneural muscle during bioassays. The hyperneural muscle reponds with sustained contracture to applications of L-glutamic acid at near $10^{-4}$ M. D-glutamic acid was much less active. The responses to glutamate are usually extremely consistent and highly reproducible. However, some preparations showed no response to L-glutamic acid even at $10^{-2}$ M, while neurally evoked responses were normal. High magnesium or low calcium salines perfused onto the preparations blocked neurally evoked contractions. The glutamate response was blocked reversibly in low calcium solutions, suggesting that the glutamate effect, when present, was presynaptic.

Dopamine, acetylcholine, 5-hydroxytryptamine, synephrine, Rogitine®, strychnine, pentobarbital, and picrotoxin, all suspected to varying degrees of some action on insect central or peripheral synaptic transmission, had no effect on the patterns of neurally evoked contracture of the hyperneural muscle.

Suspected neurotransmitter and neurohormone chemicals have been assayed on the hyperneural muscle, including: 19 of the ordinary amino acids, acetylcholine, gamma aminobutyric acid, tryptamine, 5-hydroxytryptamine (5-HT), dopamine, octopamine, synephrine, adrenaline, adenosine-triphosphate (ATP), oxytocin, and vasopressin. Besides these compounds, several blocking agents were assayed, including: strychnine, Rogitine (which contains phentolamine), and the Wellcome Research 5-HT blocker, 501 c 67 (related to xylamidine), which is shown in Figure 1. Of these, only glutamate and aspartate had any significant effect on neurally evoked contractions, and then only at $10^{-4}$ M or greater.

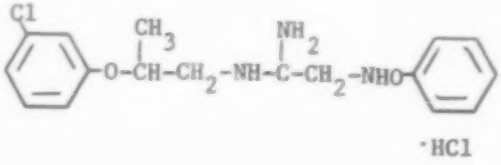


Figure 1.

Reserpine caused a gradual increase in heartbeat to 10% over control rate 3 minutes after drop-on assay of $10^{-4}$ M. When hearts were bathed in a continuous, uniform flow of $10^{-4}$ M aminophylline while preparation was maintained at a constant temperature, no pronounced changes were evident after 1½ hours. Dose-response curves of 5-HT on cockroach hearts which were pretreated with $10^{-5}$ M aminophylline were not appreciably different from control assays. It was concluded that if 5-HT were acting on adenyl cyclase in the myocardium, aminophylline did not change the 5-HT effect as might be expected.

Tyramine, tryptamine, octopamine, synephrine, 5-HT, and dopamine all acted specifically on the myocardium and not on the cardiac neurons of the cockroach heart. Threshold concentrations ranged from $5 \times 10^{-8}$ to $5 \times 10^{-7}$ M in drop-on assays. All of the compounds listed showed similar qualitative responses, and it was assumed that they were acting at a similar site on the myocardium.

A known 5-HT antagonist (Wellcome Research Laboratories 501 c 67, Fig. 1) applied to the myocardium in a range of concentrations ($10^{-6}$ M - $10^{-4}$ M) with increasing doses of 5-HT produced the parallel dose-response curves characteristic of competitive inhibition. Identical assays using this antagonist with the catecholamine, octopamine, revealed a similar pattern of reduced response. Dose ratios for antagonist - 5-HT and antagonist - octopamine were also shown to be similar, suggesting that the two antagonists act on the same receptors.

Cyclic AMP and cyclic GMP showed no obvious immediate response on myocardium or cardiac neurons, but poor solubility in saline was a problem.

L-glutamic acid, picrotoxin, reserpine, theophylline, aminophylline, prostaglandin F1, F2, and E1 were all assayed on cockroach, house fly, and noctuid moth heartbeat, but produced no appreciable immediate responses as saturated solutions or at concentrations of at least $10^{-3}$ M in the appropriate saline.

Heart preparations bathed in a continuous, uniform flow of $10^{-5}$ M dibutyryl cyclic AMP in saline while monitoring a constant temperature over a 1-hour period showed no pronounced rate change. When hearts were treated with $10^{-6}$ M 5-HT following 1 hour of exposure to cAMP, response was typical of hearts with no prior exposure to cAMP.

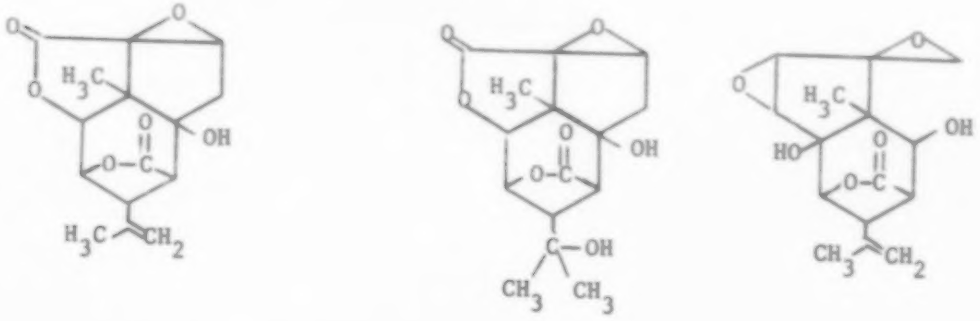
Inhibitory neuromuscular transmission - Gamma aminobutyric acid (GABA) is thought to be the inhibitory neuromuscular transmitter in arthropods. Picrotoxin and bicuculline have been found to block inhibitory neuromuscular transmission at rather low concentrations.

Compounds interfering with inhibitory synaptic transmission would be ideal models around which to develop insecticides. Blockage of inhibitory influences would free many motor pathways to fire in unison, which would be expressed as convulsions. It is known from work reported below that hyperactivity in the nervous system, if maintained a sufficient length of time, leads to central nervous damage evidenced by tissue alterations.

When applied to the isolated thoracic ganglion of house fly, picrotoxin caused characteristic convulsions in 6 min at $10^{-4}$ M. However, when applied on the desheathed ganglion, picrotoxin produced the same convulsions in 1 min at $10^{-11}$ M. By way of comparison, carbofuran, a carbamate cholinesterase inhibitor, caused similar convulsions in the desheathed ganglion after 33 min at $10^{-7}$ M. Clearly, picrotoxin has exceptionally high intrinsic toxicity to the insect central nervous system compared to carbofuran whose $LD_{50}$ to whole house flies (6.7 μg/g) is sufficient for this to be a commercial insecticide (Furadan®).

On the other hand, picrotoxin is rather unimpressive on the isolated thoracic ganglion and even less impressive on the whole fly. When applied topically at 50 μg (equivalent to a dose of 2500 μg/g), picrotoxin was nontoxic to the house fly. However, when applied with piperonyl butoxide (1:5), picrotoxin showed an $LD_{50}$ of 75 μg/g. Although this is an order of magnitude less toxic than carbofuran, it does reflect the extremely high toxicity inherent in picrotoxin and suggests that the compound is metabolized at a fairly high rate by the intact insect.

Commercial picrotoxin is a mixture of picrotin and picrotoxinin, the latter of which is thought to be the toxic component. The structure of picrotoxinin (Fig. 2a) is rather complex, with two lactone rings and an epoxide moiety. Picrotin is similar except for a hydrated double bond (Fig. 2b). Tutin (Fig. 2c) resembles picrotoxinin near the position of the double bond.



2a

2b

2c

Figure 2

Tutin was also examined for convulsant properties on the house fly thoracic ganglion. Ten µM tutin caused an initial decrease in activity, then convulsions after 52 min. 0.1 µM tutin produced convulsions in the desheathed ganglion in 35 to 40 min.

The GABA antagonist drug bicuculline shows a slow but significant hydrolysis into the pharmacologically less active substance bicucine in solutions of physiological (neutral) pH. Spectral analysis reveals that the lactone moiety of bicuculline is reversibly cleaved with a half-time of 53 min at 23° upon adjusting a stock solution at pH 3 to a pH value of 7.6 with 10 mM Tris·HCl. This occurs very rapidly, however, at 37°. Therefore, caution must be exercised in experiments using the drug in solutions at physiological pH values, especially at elevated temperatures. Solutions of bicuculline prepared at neutral pH should not be kept for any unnecessary period of time before experimentation, since misleading negative results could occur.

Bicuculline produced convulsions in the house fly thoracic ganglion at 100 µM, bicucine at 250 µM, and the other analogs were inactive at 500 µM concentrations. The evidence suggested that bicuculline was relatively less specific than picrotoxin in blocking inhibitory transmission.

On the cockroach inhibitory neuromuscular junction, bicuculline caused reversible block at 100 µM, or roughly 100 times the concentration of picrotoxin producing the same effect. Five µM bicuculline (Fig. 3a) had no effect and 50 µM was threshold, causing decreased inhibitory postsynaptic potential amplitude.

The bicuculline analogs bicucine methyl ester (Fig. 3c) and bicuculline methiodide (Fig. 3d) blocked inhibitory transmission reversibly at or near 80 µM with 40 µM thresholds, but bicucine (Fig. 3b) was inactive at 100 µM.

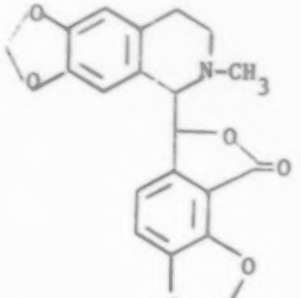
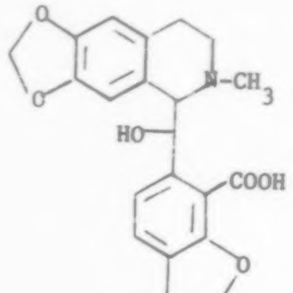


Figure 3a



3b

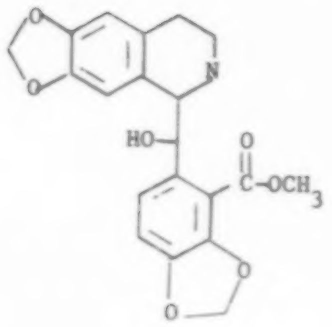
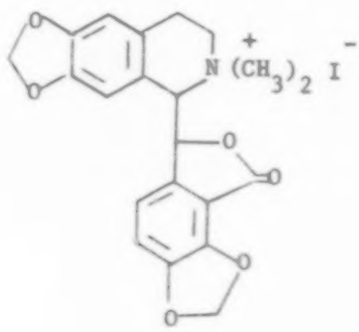


Figure 3c



3d

*Excitatory neuromuscular junction* - Excitatory transmission was examined at the insect neuromuscular junction and developed as an ultrasensitive assay for certain chemicals. A certain amount of basic work clarified the process of spontaneous release of neurotransmitter molecules, the so-called "quantal" release as measured by miniature postsynaptic potentials.

In the retractor unguis muscles of *P. americana* and *Blaberus giganteus* the rate of spontaneously occurring miniature potentials from nerve-muscle synapses associated with the "white" muscle fibers was greater than that observed in the "red" muscle fibers. In 5% of the intracellular recordings, particularly from the "white" fibers, short "bursts" of relatively high (20-100 sec$^{-1}$) rates of m.e.p.p.s. occurred infrequently in an unpredictable manner. This bursting phenomenon was not observed in extracellular recordings of the miniature potential discharge.

Rigorous statistical analyses indicated that the spontaneous mode of transmitter release at cockroach retractor unguis muscle fibers was predictable by the negative binominal theorem with a 90% reliability. Perfusion of nerve-muscle preparations with high Mg$^{2+}$ salines resulted in low spontaneous discharge rates which were characterized by a negative binomial distribution. The fit of the data to the negative binomial theorem implied some mutual interaction between those processes responsible for the spontaneous release of transmitter from cockroach nerve terminals.

Neurotomy of motor axons to skeletal muscles in insects lead to aggregation and clumping of synaptic vesicles after 48 hours. Treatment of *in vitro* nerve-muscle preparations with various respiratory poisons caused aggregation similar to that developed in neurotomized animals. This suggested that vesicle aggregation in both cases may have resulted from a decrease in available adenosine triphosphate in the nerve terminal with subsequent alteration in the normal charge density which supports a repulsive force between the vesicles.

The application of metabolic inhibitors to nerve-muscle synapses on "white" and "red" fibers in the retractor unguis muscles of *P. americana* and *B. giganteus* resulted in a dramatic increase in the spontaneous miniature potential discharge and was accompanied by a summation of the miniature potentials to form "composite" potentials. Axon terminals associated with "white" muscle fibers responded faster to metabolic inhibitors than those axon terminals associated with "red" muscle fibers. Correlated ultrastructural and electrophysiological studies inferred that a tentative relationship existed between the miniature potential activities and synaptic vesicle distributions of the nerve-muscle synapses during the phases of metabolic inhibition.

Experimentation with known metabolic inhibitors such as antimycin, oligomycin, rotenone, and salicyl anilide produced a change in the nature of the "spontaneous" miniature excitatory postsynaptic potentials recorded from the isolated metathoracic retractor unguis muscle of the cockroach, *P. americana* (Table 1). These changes consisted of an initial "bursting" of the miniature potentials, eventually cumulating to a frequency three times that in "control" or "normal" preparations. Finally, the miniature potentials decreased in frequency and ceased, with the muscle becoming electrically silent and nonresponses to electrical stimulation. This indicates that the site of action of these metabolic inhibitors is probably presynaptic.

Table 1. EFFECTS OF RESPIRATORY INHIBITORS ON SPONTANEOUS DISCHARGE OF MINIATURE POSTSYNAPTIC POTENTIALS IN NEUROMUSCULAR JUNCTION OF *P. AMERICANA* RETRACTOR UNGUIS

| | | Effect transmission | | | |
|---|---|---|---|---|---|
| Compound | | Conc. (M) | Latency (min) | Failure (min) | π Value[a] |
| I. | Antimycin $A_1$ | $10^{-7}$ | 20 | 120 | high |
| II. | Antimycin $A_1$ | $10^{-11}$ | 60 | 425 | high |
| III. | Antimycin $A_3$ | $10^{-10}$ | 15 | 55 | high |
| IV. | Rotenone | $10^{-6}$ | 10 | 50 | high |
| V. | 1,4-Napthoquinone | $10^{-6}$ | 120 | 240 | medium |
| VI. | 4-$NO_2$-methylene-dioxy benzene | $10^{-6}$ | 60 | 540 | medium |
| VII. | 4-$NO_2$-catechol | $10^{-6}$ | 45 | 210 | low |
| VIII. | 1,2-dihydroxy-9,10-anthroquinone | $10^{-6}$ | 15 | 75 | - |

[a] π value is relative octanol/water partition coefficient.

Compounds were specifically designed to interfere with the electron transport mechanism and oxidative phosphorylation processes of the mitochondrial systems of the peripheral nerve terminals. It was found that certain catechols (Table 1, VII.) were very effective at low concentrations ($10^{-6}$ M) in bringing about neuromuscular transmission failure, whereas the methylenedioxy-type compounds (Table 1, VI.) produced a reduced effect. Contrary to the latter observation, experimentation with two good methylenedioxy synergists produced neuromuscular transmission failure, while their "presumed" dihydroxy metabolite forms were even more effective. Speculation was raised as to the possible consequences of synergistic action beyond inhibition of mixed-function oxidases.

Another preparation was designed to examine the action of compounds at the cholinergic synapse in the cockroach sixth abdominal ganglion. Neurally evoked excitatory postsynaptic potentials (EPSP's) were recorded from the cercal-giant synapse in the sixth abdominal ganglion of the cockroach by the sucrose gap method. Dieldrin at $10^{-5}$ M caused an amplitude increase and prolongation of the EPSP in 30 minutes, which suggested that synaptic electrogenic currents were altered or the amount of neurotransmitter release per shock was increased.

Some controversy has arisen over the site of action of dieldrin. These results suggest that its central effects in insects may involve the presynaptic terminal of sensory axons.

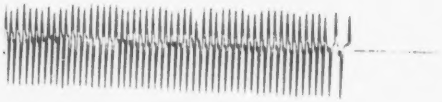
## 2. Neurophysiological Basis for Response to Insecticides

Locomotion - Behavior of house flies to poisoning was initially measured by recording locomotion. Locomotory activity of adult female house flies, *Musca domestica* L., was recorded under various conditions of light and temperature and in response to DDT application. House flies were more active at room temperature (22°C) than in the cold (16°C), as expected. Pronounced circadian rhythm was exhibited at 22°C in the laboratory associated with normal day-night conditions. No circadian rhythm patterns were evident in the cold in absence of light.

Response to lethal doses of DDT occasionally showed two peaks of locomotory activity sometimes separated by a period of depressed locomotion. Extracellular recordings of dorsal longitudinal flight muscle activity of *Musca* during DDT poisoning also showed two peaks of activity, in this case always separated by a phase of markedly depressed activity. The poisoning process in general was defined in four stages in terms of motor responses. The final stage of poisoning was redefined as tetany rather than prostration due to intensive motor unit activity which lasted for several hours, long after the cessation of convulsive movement.

*The flight motor preparation* - To observe a more sophisticated type of central nervous activity during poisoning, the output of two flight motor neurons was recorded from whole intact and tethered house flies during poisoning.

Muscle potentials recorded extracellularly from one pair of fibers of the dorsal longitudinal flight muscle in the house fly, *M. domestica*, show a very regular pattern with both pairs driven in alternating sequence (Fig. 4). House flies with chronically implanted electrodes activated the flight muscles without externally observed motion, either walking or in flight, in apparent shivering responses. The frequency of activation of the flight muscle in shivering was temperature-dependent below 25°C with $Q_{10}$ of 2.57. No shiver was recorded above 26°C.



Calibration: 20 MV, vertical;
1 sec, horizontal

Figure 4. Control of the activity of the flight muscles of *Musca*.

During insecticide poisoning, both gross locomotory activity and flight muscle activation first increased, then decreased slightly, then gradually increased again to a condition of tetany. After treatment with lethal doses of peripheral nerve poisons such as DDT and pyrethrin analogs (Fig. 5), the flight muscle activity pattern gradually increased in tetany and was constantly active for several hours. When poisoned with central nervous poisons such as Lindane,® dieldrin, endrin, and organophosphorus and carbamate anticholinesterase poisons (Figs. 6 and 7), the post-tetanic period of poisoning was characterized by discrete bursts of motor unit activation interrupted by inactivity. The high-frequency spasms of flight muscle activity during poisoning resembled hyperactivity obtained due to overheating of house flies.

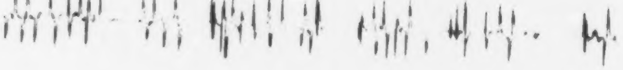


Figure 5. Flight muscle potentials 2½ hours after poisoning by 5 μg trans-Barthrin, 6-chloropiperonyl-(+)-trans-chrysanthemate. Calibration same as Fig. 4.

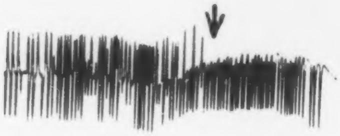


Figure 6. Flight muscle activity 10 min after treatment with 5 μg carbamate, 2,3-dihydro-2,2-dimethyl-7-benzofuranyl-N-methyl-N-thio-p-t-butylphenylcarbamate. (Arrow shows "uncoupling of motor units). Calibration same as Fig. 4.

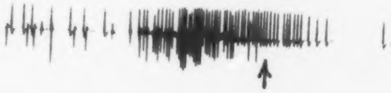


Figure 7. Flight muscle activity 6 hours after treatment with 0.2 μg of ronnel (O,O-dimethyl O-2,4,5-trichlorophenyl phosphorothioate). (Arrow shows "uncoupling" of motor units). Calibration same as Fig. 4.

Other motor units - It became obvious that when other motor units were recorded during poisoning, each class of insecticide was acting in a unique manner in terms of motor output (compare, for example, Fig. 5 with Figs. 6 and 7.).

The average heartbeat rate of female adult M. domestica was near 250 beats/min in vivo at 23°C. However, standard deviation values ranged from ±35 to ±60, depending on the individual house fly. Heartbeat rates in tethered house flies fluctuated between cessation to over 300 beats/min. The heartbeat rate was temperature-dependent with a $Q_{10}$ of 2.3. Either a bite by the Lynx spider, Peucetia vividans (Hentz), or severing the abdomen from the thorax caused the heartbeat to become extremely steady at near 300 beats/min, which gradually decreased over several minutes.

Application of lethal doses of Monitor® or Lindane to the house fly caused thoracic temperature to increase by at most 3°C in conjunction with increased convulsive activity and increased average heartbeat rate. In late stages of poisoning, the heartbeat was relatively uniform, indicating a disruption in cardioregulatory nervous activity. Response of the house fly to carbofuran or its *N*-thiomethyl analog was similar to that of Monitor and Lindane except in late stages of poisoning where the heartbeat continued to exhibit large variations in average rate.

*Speed of action of carbamate versus organophosphate insecticides* - The accumulation of $^{14}$C-labeled carbofuran, leptophos, and Monitor into the thoracic ganglion of the house fly, *M. domestica*, was measured following topical application on the abdomen or perfusion of the ganglion by the compounds in saline solution.

The penetration of $^{14}$C insecticide into the thoracic ganglion was correlated with a disruption in characteristic coupling between units of the flight motor, termed uncoupling (cf. Figs. 6 and 7). An accumulation of about 25 pg of carbofuran per thoracic ganglion was correlated with uncoupling (Fig. 8), while leptophos or Monitor did not produce uncoupling until 1000 pg had accumulated in the thoracic ganglion.

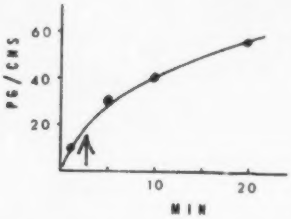


Figure 8. Amounts of radiolabel incorporated into thoracic ganglion at various times following direct contact with a wax block 9.6 ng $^{14}$C-carbofuran/g parafin. CNS = thoracic ganglion. Arrow indicates relative time to uncouple flight motor under these conditions.

It was noted that carbamate insecticides are generally fast acting as a class compared to organophosphorus insecticides whether treated topically on the whole house fly or perfused directly on the isolated thoracic ganglion. It was concluded that this general property was due to an inherent difference in the interaction of these classes of insecticide with the central nervous system.

*Difference between organophosphorus and carbamate insecticide poisoning* - Three criteria, latency to convulsions, still period, and recovery, were defined and used to distinguish between carbamate and phosphate insecticide poisoning in house flies, *M. domestica*. Because of shorter latencies with carbamate-treated flies, the central nervous system was considered more sensitive to the presence of carbamates than phosphates. Recovery from carbamate-induced tetany was considered correlated with reactivation of carbamylated cholinesterase. There appeared to be a fundamental difference between the insecticidal actions of carbamates and phosphates not wholly explainable by cholinesterase inhibition. Tethered or held house flies were less susceptible to poisoning than unrestrained flies.

## 3. Histopathology of Insecticides in House Flies

The cause of death from insecticide poisoning has not been established. It was decided to examine the thoracic ganglion of house flies and correlate tissue changes with house fly poisoning symptoms.

$S_{NAIDM}$ ♀ house flies were treated with $LD_{50}$ (6.7 $\mu g/g$) of carbofuran on the tip of the abdomen. After 24 hours, 5% of the flies behaved normally, 45% abnormally, and 50% were immobile. The abnormal flies were classified into one of the six categories: (a) unable to fly, (b) crossed front legs, (c) curled tarsi, (d) unable to walk or climb, (e) legs tucked under the thorax, or (f) rear legs extended and dragging.

All flies with abnormal behavior showed vacuolization in the neuropile of the thoracic ganglion. Thirty-three percent of the (a) flies showed vacuolization at the base of or in the dorsal nerve (flight motor nerve). The (b) flies showed vacuoles in the prothoracic region, whereas the (f) flies had lesions in the metathoracic neuromere of the thoracic ganglion.

In all abnormal flies, vacuolization was found only in the neuropile area. In the immobile (dead) flies vacuolization was extensive in all areas of the neuropile. Thus a good correlation existed between central lesions produced by carbofuran and ultimate behavior of the house flies.

Lindane, dieldrin, pyrethrins, and DDT were also treated on the $S_{NAIDM}$ strain at $LD_{50}$ values. Thirty percent of the Lindane-treated flies survived, and of these two-thirds showed no lesions in the thoracic ganglion and no abnormal behavior. The remaining one-third were unable to fly and had vacuolization in the glial or perineurial layer of the thoracic ganglion. All of the Lindane-poisoned flies which were classed as dead had normal-appearing neuropile with general lesions in the perineurium.

Those flies treated with $LD_{50}$ of dieldrin which showed abnormal behavior after 24 hours had vacuolization in all areas of the thoracic ganglion plus lesions in the perineurial area of the brain. Of the apparently normal survivors, one-third showed slight vacuolization in glia and perineurium of the thoracic ganglion.

Pyrethrins caused general vacuolization in thoracic ganglia of house flies which did not survive. All 24-hour survivors had little vacuolization in the thoracic ganglia despite stereotyped abnormal behavior including inability to fly, rear legs contracted under the thorax, and improper landing from flight. Of these survivors, some vacuolization of the optic ganglion was noted.

## 4. References

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## M. INSECT RESISTANCE TO CHEMICALS: RESISTANCE TO INSECT GROWTH REGULATORS

### 1. Cross Resistance to Juvenile Hormone Analogs in Insecticide-Resistant Strains of the House Fly

Introduction - It is now well known that juvenile hormones (JH) play an important role in insect growth and development. It is also generally acknowledged that synthetic juvenile hormone analogs (JHA) hold promise as insect control agents. One attractive aspect of these compounds was the suggestion by Williams[1] that insects could not develop resistance to them. However, the results described below,[2,3] on the presence of cross resistance to JHAs in insecticide-resistant strains of the house fly have introduced an element of caution in these predictions.

Materials and Methods - The strains tested comprised one susceptible (NAIDM-S), one organochlorine-resistant (DDT/lindane-R), a carbamate-resistant (OMS-15-R, selected by m-isopropylphenyl methylcarbamate), a field strain (SK-R) colonized in 1971, and five organophosphorus-resistant [parathion-R; dimethoate-R; fenthion-R; Chlorthion-R, selected by O,O-dimethyl O-(3-chloro-4-nitrophneyl) phosphorothioate; and OMS-12-R, selected by O-ethyl O-(2,4-dichlorophenyl) phosphoramidothioate]. With the exception of NAIDM-S and SK-R strains, all have been under specific selection pressure in the laboratory for more than 10 years, and each is believed to have attained maximal resistance to the selecting insecticide. The levels of resistance in the various strains towards their selecting compounds are: DDT/lindane-R >1000x; parathion-R >1643x; dimethoate-R 60x; fenthion-R 435x; Chlorthion-R >430x; OMS-12-R 88x; and OMS-15-R >100x. In all instances, the resistance is highest towards the selecting compound, although each strain demonstrates various levels of cross resistance to other compounds as well. The SK-R strain was reported to possess the following levels of resistance at the $LD_{95}$ to organophosphorus insecticides: diazinon, 580x; ronnel, 737x; malathion, >100x; fenthion, 43x; naled, 26x; and dimethoate, 15x.[4]

The chemical structures of the compounds tested were as follows:

I. Methoprene (Altosid®): isopropyl 11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate, a mixture of isomers, supplied by Zoecon Corporation, Palo Alto, Ca.
II. Ro 7-9767: 6,7-epoxy-3,7-diethyl-1-(3,4-(methylenedioxy)phenoxy)-2-cis/trans-octene, a mixture of isomers, supplied by Hoffmann-LaRoche Inc., Nutley, New Jersey.
III. R-20458: trans 1-(4'-ethylphenoxy)-6,7-epoxy-3,7-dimethyl-2-octene, supplied by Stauffer Chemical Co., Mountain View, Ca.
IV. NIA 23509: 10,11-epoxy-N-ethyl-3,7,11-trimethyl-2,6-dodecadienamide, a mixture of isomers supplied by FMC Corp., Princeton, New Jersey.

V. Sesamex: acetaldehyde 2-(2-ethoxy-ethoxy) ethyl 3,4-methylene dioxyphenyl acetal, supplied by the World Health Organization.

Acetone was used in making all weight/volume dilutions. All chemicals, both neat and diluted, were stored at 0°C in the dark. Solutions were kept for one month at most and were remade if further tests were required.

White prepupae, a stage lasting at most 1 h at 24°C and known to be the most sensitive to exogenous JH, were treated by the application of 0.5 µl of JHA solution. Treated prepupae in batches of ten were placed in unwaxed 8 oz squat paper cups. The cups were covered with nylon netting and stored under a 12:12 h photoperiod at 27°C. Relative humidity was 60% during photophase and 95% during scotophase. Effects were assessed 7 days after treatment.

Activity of the compounds was based on the number of flies successfully completing emergence from the puparium. Eclosed adults failing to free themselves completely from the puparium were scored as affected. Such flies showed the presence of varying degrees of morphogenetic damage characteristic of juvenile hormone action: deformation of one or both wings, or lack of wing expansion were common defects; the genitalia of both sexes were poorly developed and in some males they failed to complete the 180° rotation; misshaped ventral arches of the fifth and sixth abdominal segments, and abnormally formed primary forceps, were also found in some males.

Each replicate consisted of ten insects. In most instances, a total of ten replicates/dose were run, each on a different day. Acetone-treated controls were maintained for each test. Cross resistance determinations were performed mostly at two dose levels, chosen to produce 60% and 90% effect against the S strain. Duncan's multiple range test was used to rank the mean responses of the strains to the JHAs at the 95% confidence level. Probit analysis was used in determining dose-response regression lines for the responses of the S and certain R strains towards the compounds.

Results - Dose-response data summarized in Table 1 indicate that the JHAs tests varied considerably in their activity toward S house flies. Methoprene was by far the most active compound tested ($ED_{50}$: 0.0033 µg/prepupa; $ED_{95}$: 0.00634 µg/prepupa), producing its effect over a narrow range of doses, as shown by the relatively high slope value ($\underline{b}$=5.8). The other compounds were much less active and the slope of the regression lines became increasingly lower with loss of activity. The least active compound tested, NIA 23509, was 10,200 times less active than methoprene at $ED_{50}$.

Table 1. COMPARATIVE ACTIVITY OF VARIOUS JUVENILE HORMONE ANALOGUES ON THE NAIDM SUSCEPTIBLE STRAIN OF HOUSE FLY

| Compound | $ED_{50}$[a] (μg/prepupa) | Slope | Fold decrease in activity at $ED_{50}$ |
|---|---|---|---|
| Methoprene | 0.0033<br>(0.00302-0.0036)[b] | 5.8 | 1 |
| Ro 7-9767 | 0.325<br>(0.142-0.581) | 2.0 | 98 |
| R-20458 | 5.57<br>(4.63-6.69) | 1.2 | 1,690 |
| Sesamex | 33.5<br>(28.0-40.2) | 1.5 | 10,200 |
| NIA 23509 | 33.6<br>(20.7-65.1) | 1.2 | 10,200 |

[a] Dose for 50% effect.

[b] Upper and lower fiducial limits.

The ranking of cross resistance of the nine house fly strains to methoprene was obtained by treatment with 0.025 μg/prepupa of the JHA, a dose approximately 2.7 times the minimum required to produce complete suppression of adult emergence in the NAIDM-S strain. The dimethoate-R strain was the most resistant one tested (Table 2). OMS-15-R and OMS-12-R strains showed a high level of cross resistance, while the fenthion-R strain was intermediate. The Chlorthion-R strain had only weak resistance to methoprene and the SK-R, parathion-R, and DDT/lindane-R strains showed minimal resistance.

Subsequent tests were run to examine in greater detail the susceptibility of the dimethoate-R, fenthion-R, and SK-R strains to methoprene. The results in Table 3 show an increased homogeneity of response (b=2.8) and a relatively low level of cross resistance to methoprene (2.55x at $ED_{50}$) in the SK-R field strain. Greater homogeneity of response (b=1.9) was found in the two laboratory-stressed strains along with increased resistance levels, i.e. 5.5x in fenthion-R and 39.4x in dimethoate-R. The resistance of these strains is fully evident when the relative resistance ratios are examined at the practical control level of $ED_{95}$, *viz*. SK-R 5.1, fenthion-R 17.5, and dimethoate-R 150.8

Table 2. RESPONSE OF VARIOUS STRAINS OF THE HOUSE FLY TO TREATMENT WITH METHOPRENE AT 0.025 μg/PREPUPA

| Strain | Prepupae treated | Percentage mortality[a] ± $S_{\bar{x}}$ |
|---|---|---|
| NAIDM-S | 100 | 100.0[a] |
| DDT/lindane-R | 100 | 97.0 ± 1.53[a] |
| Parathion-R | 100 | 91.0 ± 3.48[ab] |
| SK-R | 100 | 89.3 ± 4.38[ab] |
| Chlorthion-R | 100 | 80.3 ± 5.79[b] |
| Fenthion-R | 100 | 56.4 ± 4.23[c] |
| OMS-15-R | 100 | 28.0 ± 4.16[d] |
| OMS-12-R | 100 | 27.2 ± 5.08[d] |
| Dimethoate-R | 100 | 12.0 ± 4.42[e] |

[a] Any two means having the same superscript are not significantly different at the 5% level as determined by Duncan's multiple range test.

Table 3. RESPONSE OF FOUR STRAINS OF THE HOUSE FLY TOWARD METHOPRENE

| Strain | $ED_{50}$[a] μg/prepupa | $ED_{95}$[a] μg/prepupa | Slope (b) | Resistance at $ED_{50}$ | Ratio[b] at $ED_{95}$ |
|---|---|---|---|---|---|
| NAIDM-S | 0.0033 | 0.0063 | 5.8 | | |
| SK-R | 0.0034 | 0.032 | 2.8 | 2.5 | 5.1 |
| Fenthion-R | 0.018 | 0.11 | 1.9 | 5.5 | 17.5 |
| Dimethoate-R | 0.13 | 0.95 | 1.9 | 39.4 | 150.8 |

[a] Dose for 50% or 95% effect.

[b] ED of R strain ÷ ED of S strain.

The cross resistance data obtained on the various strains utilizing compounds Ro 7-9767, R-20458, Sesamex, and NIA 23509 are given in Table 4. The overall picture emerging from all tests was as follows: the dimethoate-R and OMS-15-R strains appeared to exhibit a generally high level of resistance to all the JHAs tested. The fenthion-R strain showed high resistance levels to all the compounds but methoprene, towards which it exhibited an intermediate level of 17.5x. Similarly, the DDT/lindane-R strain manifested only negligible tolerance to methoprene, but an intermediate response to all the other compounds. The OMS-12-R strain demonstrated intermediate to high levels of cross resistance to all JHAs tested, and the SK-R, parathion-R, and Chlorthion-R strains showed, for the most part, a relatively low to intermediate level of resistance.

Discussion - The varying degrees of juvenile hormone activity of the compounds tested against the susceptible NAIDM flies are most likely due to differences in the intrinsic juvenile hormone activity of the chemicals and their metabolic stability. Increased intrinsic activity could result from a sterochemical structure better suited for function at the target site of the natural JH.

Metabolic stability may also be an imporant factor: it has been suggested that not all cells in the insect become sensitive to JH at the same time so that the longer a JHA remains intact, the more likely it is to contact each cell during the sensitive period.[5]

The metabolic stability of methoprene may possibly explain its relatively high activity on susceptible flies as compared to the other compounds. Studies of the inactivation of the natural juvenile hormones in various species have established that metabolism proceeds primarily by esterase attack at the carboxymethyl moiety and by epoxide hydration.[6-8] Methoprene lacks the epoxide moiety, and a recent study suggests that the terminal isopropyl group may provide protection from carboxyesterase degradation.[9] The other compounds, possessing epoxy groups or unprotected esters, may more easily be inactivated.

Finding cross resistance to JHAs was not entirely unexpected. Inactivation sites, present in these compounds[7-9] are likely targets for the degradative metabolic systems of insecticide-resistant strains.

The varying spectrum of cross resistance to the JHAs in each strain would suggest that several degradative mechanisms may be involved in resistance. In light of the large variability in the ranking responses of the organophosphorus-resistant strains, phosphotriesterase activity, common in OP-resistant insects, may be of minor importance in cross resistance to JHAs.

It appears that mixed function oxidases play an important role in the deactivation of JHAs. The OMS-15-R (carbamate-resistant) strain, known

Table 4. RESPONSE OF VARIOUS STRAINS OF THE HOUSE FLY TO TREATMENT OF PREPUPAE WITH JHAs

| Strains | Percentage mortality at doses indicated$^{a}$ | | | |
|---|---|---|---|---|
| | Ro 7-9767 | R-20458 | Sesamex | NIA 23509 |
| | 0.5 μg/prepupa | 10 μg/prepupa | 50 μg/prepupa | 50 μg/prepupa |
| NAIDM-S | 52.8 ± 7.69$^{a}$ | 62.1 ± 5.40$^{a}$ | 65.9 ± 6.83$^{a}$ | 59.0 ± 6.40$^{a}$ |
| Chlorthion-R | 15.8 ± 6.25$^{b}$ | 52.5 ± 5.65$^{ab}$ | 40.5 ± 10.19$^{b}$ | 36.1 ± 7.66$^{bc}$ |
| SK-R | 12.1 ± 3.26$^{b}$ | 53.7 ± 3.54$^{ab}$ | 46.3 ± 5.11$^{b}$ | 37.9 ± 7.26$^{bc}$ |
| Parathion-R | 16.2 ± 4.73$^{b}$ | 34.4 ± 7.39$^{bc}$ | 36.9 ± 8.68$^{b}$ | 23.2 ± 4.42$^{bcd}$ |
| DDT/Lindane-R | 14.0 ± 6.53$^{b}$ | 35.5 ± 4.57$^{bc}$ | 34.2 ± 5.36$^{b}$ | 35.0 ± 6.54$^{bc}$ |
| OMS-15-R | 6.3 ± 2.38$^{b}$ | 5.0 ± 1.67$^{e}$ | 12.5 ± 4.71$^{c}$ | 11.3 ± 3.54$^{d}$ |
| OMS-12-R | 4.1 ± 3.08$^{b}$ | 29.0 ± 5.07$^{cd}$ | 8.2 ± 2.03$^{c}$ | 41.5 ± 6.34$^{b}$ |
| Fenthion-R | 11.0 ± 2.77$^{b}$ | 12.4 ± 5.68$^{de}$ | 13.3 ± 2.20$^{c}$ | 20.6 ± 3.53$^{cd}$ |
| Dimethoate-R | 5.1 ± 2.26$^{b}$ | 8.2 ± 5.18$^{de}$ | 15.5 ± 4.18$^{c}$ | 27.9 ± 6.19$^{bcd}$ |
| | 2 μg/prepupa | 100 μg/prepupa | 200 μg/prepupa | 200 μg/prepupa |
| NAIDM-S | 97.0 ± 2.13$^{a}$ | 97.0 ± 2.14$^{a}$ | 85.9 ± 2.69$^{a}$ | 72.0 ± 3.59$^{a}$ |
| Chlorthion-R | 65.6 ± 7.04$^{b}$ | 90.7 ± 3.60$^{a}$ | 45.2 ± 8.26$^{c}$ | 56.1 ± 8.75$^{b}$ |
| SK-R | 64.4 ± 4.03$^{b}$ | 94.3 ± 2.57$^{a}$ | 71.1 ± 4.59$^{b}$ | 55.1 ± 5.36$^{bc}$ |
| Parathion-R | 60.3 ± 4.01$^{b}$ | 89.5 ± 3.18$^{a}$ | 67.8 ± 6.85$^{b}$ | 51.8 ± 5.10$^{bc}$ |
| DDT/Lindane-R | 38.0 ± 4.90$^{c}$ | 74.6 ± 5.03$^{ab}$ | 51.6 ± 5.22$^{c}$ | 54.0 ± 4.27$^{bc}$ |
| OMS-15-R | 13.4 ± 3.70$^{d}$ | 35.5 ± 5.42$^{d}$ | 24.5 ± 4.89$^{d}$ | 44.3 ± 5.26$^{bcd}$ |
| OMS-12-R | 12.7 ± 4.70$^{d}$ | 61.5 ± 2.83$^{bc}$ | 15.0 ± 4.28$^{d}$ | 53.7 ± 3.17$^{bc}$ |
| Fenthion-R | 12.0 ± 2.00$^{d}$ | 52.4 ± 5.83$^{bcd}$ | 29.3 ± 2.91$^{d}$ | 34.9 ± 3.76$^{d}$ |
| Dimethoate-R | 7.1 ± 4.70$^{d}$ | 44.0 ± 4.01$^{cd}$ | 25.6 ± 3.06$^{d}$ | 40.1 ± 3.60$^{cd}$ |

[a] Average percentage mortality ± $S_{\bar{x}}$ based on 100 prepupae/dose after correction for control mortality by Abbott's formula. Any two means having the same superscript are not significantly different at the 5% level as determined by Duncan's multiple range test.

to metabolize carbamates by MFO action, showed high levels of cross resistance to all the JHAs. This is in agreement with the results of Terriere and Yu[9] that methoprene metabolism in a housefly strain possessing a high oxidase content proceeds at more than twice the rate found in a susceptible strain with a low oxidase content. Likewise, a study by Vinson and Plapp[10] showed that cross resistance to certain JHAs in the house fly is controlled by genetic factor(s) in linkage group II, which also controls high levels of oxidase activity.

## 2. Cross Resistance to Chitin Synthesis Inhibitors in Insecticide-Resistant Strains of the House Fly

Introduction - Among various synthetic insect growth regulators investigated in recent years are included the benzoylphenylureas[11] whose action resides in their ability to inhibit the synthesis and deposition of chitin, thereby causing death of developing insects.[12] Insecticidal activity has been reported against a large number of insect species.

One major problem facing every potential insecticide is the possibility of development of resistance to it by the target pest. We have therefore investigated the presence of cross resistance to the most potent known member of this class of compounds, Dimilin® [1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea] in certain insecticide-resistant strains of the house fly.[13]

Materials and Methods - The strains utilized and the methods of tests were the same as described in Section 1, with the exception of the use of tetrahydrofuran as solvent due to the low solubility of Dimilin in acetone.

Results - The visible response of the treated insects was failure to emerge from the puparium, ranging from minimal leg attachment to complete lack of emergence.

Base-line data for the NAIDM-S strain showed a modest $ED_{50}$ of 0.265 μg/prepupa and a relatively high $ED_{95}$ of 70 μg/prepupa. The low slope of the regression line ($\underline{b}$=0.68) may indicate a slow rate of penetration through the cuticular layer.

Various levels of cross resistance to Dimilin by the eight insecticide-resistant strains of the house fly were revealed by the application of the compound at two dosage levels, e.g. 1 and 10 μg/prepupa (Table 5). The low dose provided ranking separation only between NAIDM-S and the resistant strains. But 10 μg/prepupa provided significant separation of the strains into three groups: the parathion-R and Chlorthion-R strains were the least resistant, the DDT/lindane-R, fenthion-R, OMS-15-R, dimethoate-R, and SK-R strains were intermediate, while the OMS-12-R was the most resistant of all strains tested.

Table 5. SUSCEPTIBILITY OF VARIOUS STRAINS OF THE HOUSE FLY TO DIMILIN AT DOSES OF 1 μg AND 10 μg PER PREPUPA[a]

| Strain | 1 μg/prepupa | | 10 μg/prepupa | |
|---|---|---|---|---|
| | % mortal. | $S_{\bar{x}}$ | % mortal. | $S_{\bar{x}}$ |
| NAIDM-S | 60 | $5.96^{a}$ | 87 | $4.73^{a}$ |
| Parathion-R | 32.3 | $6.39^{b}$ | 63.3 | $3.48^{b}$ |
| Chlorthion-R | 28.3 | $7.93^{b}$ | 58.6 | $5.37^{b}$ |
| DDT/Lindane-R | 21 | $5.18^{b}$ | 42 | $6.38^{c}$ |
| Fenthion-R | 20.9 | $6.01^{b}$ | 35.6 | $6.62^{c}$ |
| OMS-15-R[b] | 25 | $6.19^{b}$ | 32.4 | $5.28^{cd}$ |
| Dimethoate-R | 18.5 | $3.84^{b}$ | 32.3 | $6.18^{cd}$ |
| SK-R | 17.3 | $5.27^{b}$ | 28.1 | $4.12^{cd}$ |
| OMS-12-R[c] | 15.2 | $4.98^{b}$ | 18.5 | $4.95^{d}$ |

[a] Based on 100 insects per test. Any two means having the same superscript letter are not significantly different at the 5% level by Duncan's multiple range test.

[b] *m*-Isopropylphenyl methylcarbamate.

[c] *O*-Ethyl *O*-(2,4-dichlorophenyl)phosphoramidothioate.

Previous reports have indicated that the benzoylphenylureas act only as stomach poisons.[14,15] However, by topically applying Dimilin in tetrahydrofuran to white prepupae, we have demonstrated that entry via the stomach route is not an essential requirement for toxicity.

Discussion - The relatively high levels of cross resistance toward Dimilin demonstrated by this study may be disconcerting. Extrapolation of susceptibility data for the strain with the lowest resistance, parathion-R, reveals the presence of tolerance of approximately ten-fold at the $ED_{50}$. Resistance in the OMS-12-R strain is obviously several fold greater. It is especially significant that the field strain, SK-R, is among the most resistant toward Dimilin. The detection of high levels of resistance in the house fly serves to emphasize the need for judicious use of new chemicals against presently susceptible populations, under conditions which minimize the degree of selection pressure.

## 3. Genetic Basis of Cross Resistance to Juvenile Hormone Analogs

Introduction - Since the above studies (Section 1) have indicated a relationship between resistance to certain insecticides and cross resistance to JHAs, a study was undertaken to establish the genetic basis of this relationship. If linkage between factors responsible for insecticide resistance and JHA cross resistance could be demonstrated, this would enhance the hypothesis for co-identity of these phenomena.

Materials and Methods - Dose-response assays were carried out with methoprene on a ronnel-selected strain (R), a susceptible marker strain (boaa), and on "sister" strains in which single pairs of chromosomes of the R strain had been incorporated. The genetic method utilized and the properties of these strains have been described.[16]

Results and Discussion - The regression lines obtained with each population are given in Figure 1, and the calculated resistance ratios are presented in Table 6. Despite the low resistance present in the parental strains (e.g. 6.3x), it is clear that the greatest share of resistance is contributed by factors on chromosome 2. Chromosome 3 by itself does not contribute detectable resistance, but in combination with chromosome 2 it evidently acts as an enhancer of resistance. This is in accord with our earlier investigations which established that resistance to ronnel is conferred mainly by chromosome 2 and that the effects of chromosomes 2 and 3 are synergistic.

Table 6. GENETIC ANALYSIS OF RESISTANCE TO METHOPRENE IN A RONNEL-SELECTED STRAIN OF HOUSE FLY

| Strain | $LC_{50}$ (%) | $LC_{95}$ (%) | Resistance ratio[a] at $LC_{50}$ | Resistance ratio[a] at $LC_{95}$ |
|---|---|---|---|---|
| Parental strains | | | | |
| Susceptible (boaa) | $.46 \times 10^{-4}$ | $2.0 \times 10^{-4}$ | | |
| Resistant (ronnel) | $2.9 \times 10^{-4}$ | $8.3 \times 10^{-4}$ | 6.3 | 4.2 |
| Derivative strains | | | | |
| R chromosome II | $1.8 \times 10^{-4}$ | $5.6 \times 10^{-4}$ | 3.9 | 2.8 |
| " III | $.3 \times 10^{-4}$ | $1.2 \times 10^{-4}$ | .7 | .6 |
| " II + III | $2.9 \times 10^{-4}$ | $10 \times 10^{-4}$ | 6.3 | 5.0 |

[a] Resistance ratio = $\frac{LC_{50} \text{ of R or derivative strain}}{LC_{50} \text{ of S strain}}$

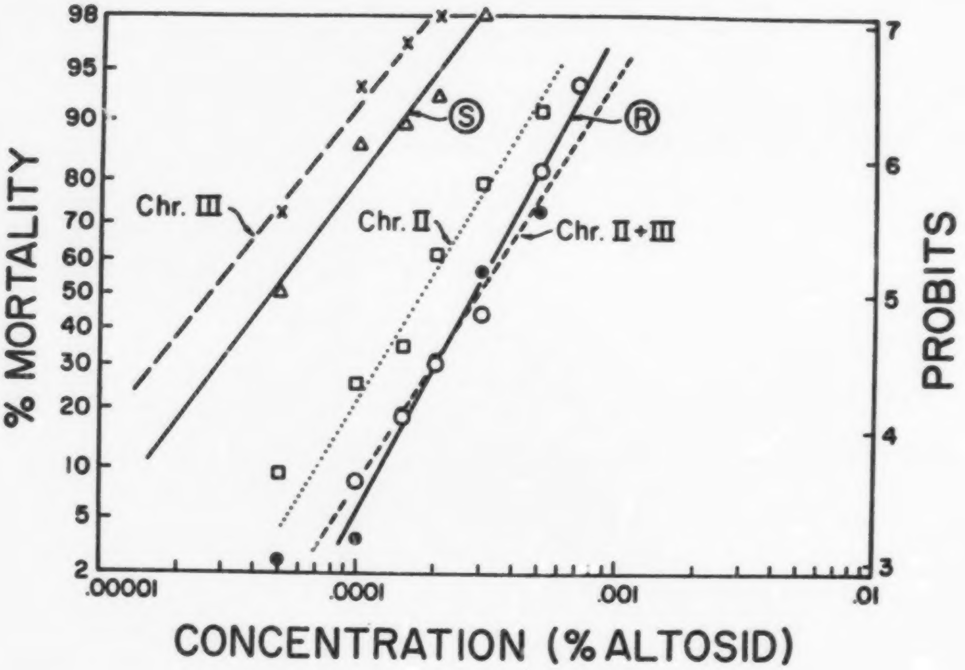

Figure 1. Dose-mortality relationships of R, S, and derivative strains (with chromosomes II, III, and II + III) established by exposing late 4th instar larvae to methoprene-treated media.

## 4. Development of Resistance by Specific Selection with Methoprene and Characteristics of Resistance

Introduction - Although the presence of cross resistance to JHAs in insecticide-resistant strains was clearly demonstrated, the possibility of development of resistance by specific selection by these materials remained unknown. It was, therefore, considered desirable to initiate selections by a representative compound, methoprene, and to subsequently carry out a comprehensive study of the resistance which might result.

Materials and Methods - The study involved treatment of successive generations of two strains (Dimethoate-R and field SK) by partially lethal doses of methoprene. A sensitive selection method was developed involving exposure of late 4th instar larvae to a pupation medium impregnated with the required amount of methoprene. Data were obtained on the extent of pupal eclosion, adult fecundity, and egg fertility. A second series of selections (Series II) on the same parental strains was also carried out in order to test the results obtained in Series I.

Results and Discussion - In conjunction with selection for resistance the comparative biological and biotic effects of methoprene on house flies were investigated. The effects of topical treatment of prepupae included non-emergence or partial emergence of adults, deformed and non-functional wings, incomplete development of genitalia, incomplete rotation of male genitalia, and "fatal drunkeness." Longevity, fecundity and fertility were assayed quantitatively following treatment of dimethoate-R and NAIDM-S strains at the "maximum sublethal" and $ED_{50}$ levels. No effects were noted at the sublethal doses, but at the $ED_{50}$ the post-eclosion biotic potential of both strains as expressed by fertility and fecundity was severely impaired.

Selection of the two strains by methoprene produced a rapid increase in resistance to this compound (Table 7, Figure 2). In the dimethoate-R strain, resistance rose from an initial level of 39.4-fold to 1188-fold at the completion of 30 generations of selection in Series I and 6060-fold at the completion of 18 generations of selection in Series II. Similar selection of strain SK resulted in an increase in resistance from an initial level of 2.5-fold to 1273-fold in Series I and 112-fold in Series II (Table 7). Substantially high resistance was also evident when susceptibility to methoprene was determined by exposure of 4th instar larvae to treated media, instead of by topical application to white prepupae (Table 8, Figure 3).

The above large increases in resistance were also accompanied by a substantial reduction in oogenetic disturbances in the adult insect following exposure to the chemical.

The effect of development of resistance to methoprene on cross resistance toward other JHAs was also determined. The strains under investigation showed increases in cross resistance toward the JHAs Ro 7-9767 and

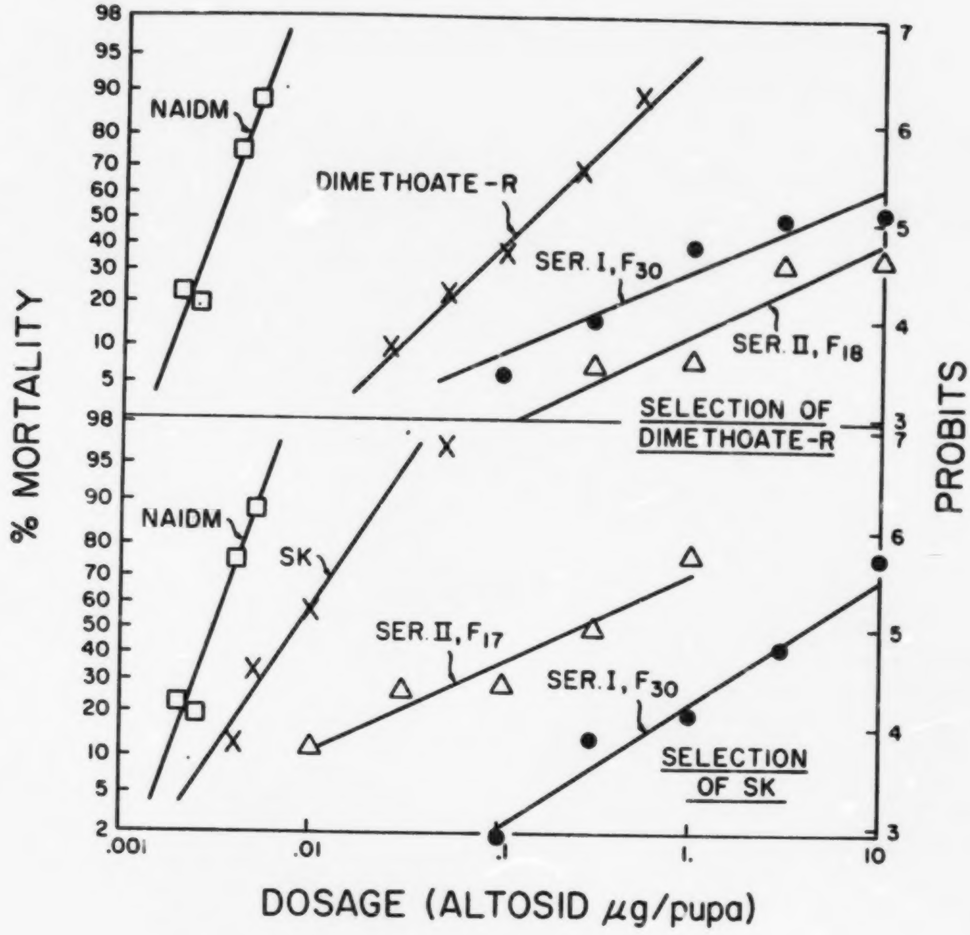

Figure 2. Development of resistance to methoprene (Altosid) in dimethoate-R (top) and SK (bottom) strains of the house fly in two series of selections, I and II. Assays performed by topical application to white prepupae.

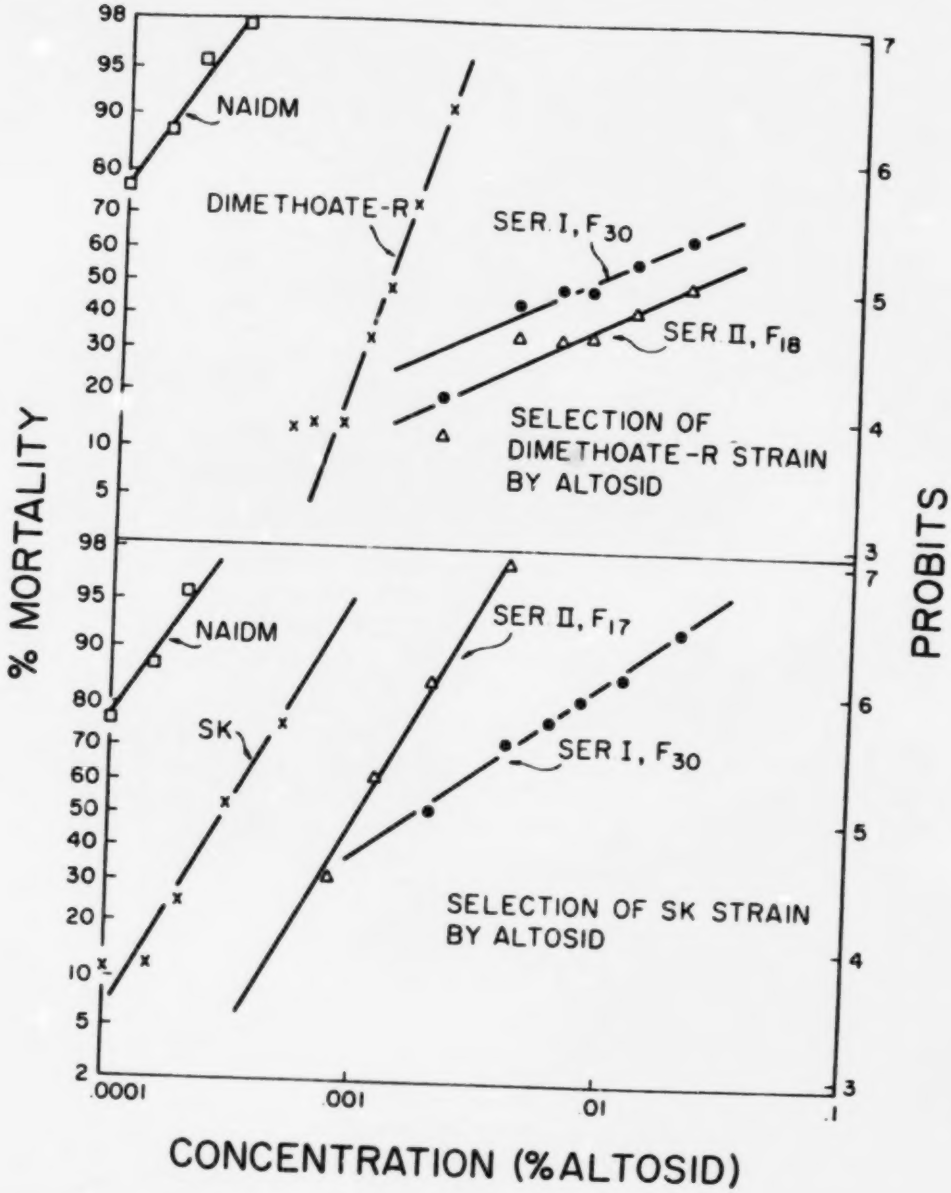

Figure 3. Development of resistance to methoprene (Altosid) in dimethoate-R (top) and SK (bottom) strains of the house fly in two series of selections I and II. Assays performed by exposure of 4th instar larvae to treated media.

Table 7. EFFECT OF METHOPRENE SELECTION PRESSURE ON CROSS RESISTANCE TO INSECTICIDES IN THE HOUSE FLY

| Compound | S-NAIDM $ED_{50}$ $\gamma$/prepupa | Resistance ratio[a] SK-R P | SK-R Ser. I $F_{30}$ | SK-R Ser. II $F_{17}$ | Dimethoate-R P | Dimethoate-R Ser. I $F_{30}$ | Dimethoate-R Ser. II $F_{18}$ |
|---|---|---|---|---|---|---|---|
| Methoprene | .0033 | 2.5 | 1273. | 112. | 39.4 | 1188. | 6060. |
| Dimethoate | .011 | 3.8 | 6.0 | 8.5 | 103.6 | 23.6 | 40. |
| OMS-12 | .072 | 6.0 | 15.7 | 26.4 | 18.5 | 15.3 | 18.1 |
| Parathion | .024 | 59.2 | 118.3 | - | 16.7 | 63.8 | - |
| Fenthion | .043 | 10.9 | 29.0 | - | 93.0 | 50.7 | - |
| OMS-15 | 1.3 | 3.1 | (30%) | (20%) | (0%) | (2.5%) | (2%) |
| Aldrin | .058 | 5.0 | 1.1 | 1.9 | - | 2.1 | 2.1 |
| Dieldrin | .008 | 4.3 | 6.5 | - | 12.5 | 10.9 | - |

[a] Resistance ratio = $\frac{ED_{50} \text{ of selected strain}}{ED_{50} \text{ of parental strain}}$

Table 8. DEVELOPMENT OF RESISTANCE TO METHOPRENE BY SELECTION OF A DIMETHOATE-RESISTANT AND A FIELD (SK) STRAIN OF HOUSE FLY

| Strain | $EC_{50}$ (%) | $EC_{95}$ (%) | Resistance ratio[a] at $EC_{50}$ | Resistance ratio[a] at $EC_{95}$ |
|---|---|---|---|---|
| NAIDM | $.54 \times 10^{-4}$ | $2.2 \times 10^{-4}$ | | |
| Parental SK | $3.1 \times 10^{-4}$ | $9.5 \times 10^{-4}$ | | |
| Ser. I, $F_{30}$ | $16 \times 10^{-4}$ | $28 \times 10^{-3}$ | 5.2 | 29.5 |
| Ser. II, $F_{17}$ | $11 \times 10^{-4}$ | $32 \times 10^{-4}$ | 3.5 | 3.4 |
| Parental Dimethoate | $12 \times 10^{-4}$ | $23 \times 10^{-4}$ | | |
| Ser. I, $F_{30}$ | $72 \times 10^{-4}$ | .57 | 6.0 | 247.8 |
| Ser. II, $F^{18}$ | $18 \times 10^{-4}$ | 1.17 | 15.0 | 508.7 |

[a] Resistance ratio = $\frac{EC \text{ of selected strain}}{EC \text{ of parental strain}}$

R-20458. In general the magnitude of increase was positively correlated with the intrinsic activity of the compounds against the susceptible strain. Thus the degree of change in strain SK for methoprene, Ro 7-9767 and R-20458 ($ED_{50}$ 0.017, 2.6 and 49 μg/pupa, respectively) was 6060x, 1545x, and 12.3x, respectively.

Further studies concerned the effect of selection by methoprene on the insecticide-resistance levels of the above strains (Table 7). In general only small changes in the cross resistance spectrum (less than 3x) were noted, although resistance to methoprene itself had increased to a 1273x level in strain SK and 6060x level in strain dimethoate-R. The most pronounced chance in cross resistance was toward fenthion, which rose from an initial level of 10.9x before methoprene selection in strain SK to 29x after 30 generations of pressure. In the same strain there were small increases in cross resistance toward dimethoate (1.6x), OMS-12 (2.6x), dieldrin (1.5x), and parathion (2.0x). In strain dimethoate-R there was an increase in cross resistance to parathion (3.8x), but small reductions in cross resistance to dimethoate (4.4x), OMS-12 (1.2x), fenthion (1.8x), and dieldrin (1.1x). These results indicate a random drift in the cross resistance pattern of the strains and suggest that the induced resistance toward the juvenile hormone analog may be due to factors unrelated to insecticide resistance.

The possibility of reduced penetration as a mechanism of resistance to JHAs was examined by the use of radiolabeled R-20458. Sublethal doses of this JHA were applied topically to white prepupae and allowed to penetrate for various time intervals between 0 and 720 minutes, and the radioactivity was determined at the end of each period. The results revealed that the JHA penetrated somewhat more slowly in susceptible than in resistant pupae, especially during the 90-360 minute period. Thus a reduction in the rate of penetration, as is usually observed in cases of insecticide resistance, was not found to be a factor in resistance to the JHA.

In view of the similarity in the results of selection in the two series, only the strains in Series I have been retained for further study on the mechanisms of resistance. Beyond generation 30, resistance appears stable and is being maintained by treatment of pupation medium at a concentration of 200 ppm methoprene. Such exposure provides approximately 60% pupal mortality and 73% egg sterility, i.e. a total pressure of 89%.

## 5. Selection of House Flies for Resistance to Dimilin

Introduction - In view of our earlier finding that insecticide-resistant house flies possess various degrees of cross resistance to the chitin synthesis inhibitor Dimilin,[13] a strain (dimethoate, methoprene-resistant) was placed under selection pressure by Dimilin in order to enhance and concentrate the existing resistance factors for further study. Interest in Dimilin as a potential insecticide has been enhanced by the demonstration of activity of a high order against a variety of pest species in several insect families and orders.

*Materials and Methods* - The selection procedure developed for this purpose involves the incorporation of Dimilin in the larval medium, beginning with the first instar of larval growth, and assessment of results on the basis of adult emergence, fecundity, and fertility.

*Results and Discussion* - Following selection for 4 generations at 2 ppm Dimilin and of 6 additional generations at 3 ppm, suppression of adult emergence declined from 96.4% in $F_1$ to 87.6% in $F_{10}$. Concurrently, egg fertility increased from 58.2% in $F_1$ to 72.2% in $F_{10}$. In the $F_{20}$ selected generation the strain could tolerate 0.125 ppm Dimilin in the rearing medium, suffering a reduction in adult yield of approximately 75%. This effect is equivalent to approximately 8x increase in resistance over the level present in the parental (Dimethoate-R) strain.

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## N. INSECT RESISTANCE TO CHEMICALS: INSECTICIDE RESISTANCE IN MOSQUITOES

### 1. Resistance to Organophosphates and Carbamates in *Anopheles albimanus* Based on Reduced Sensitivity of Acetylcholinesterase

*Introduction* - One of the outstanding cases of resistance in recent years has been that of *Anopheles albimanus*, the principal vector of malaria in Central America, which has become resistant to a large variety of organophosphate and carbamate insecticides.[1,2] Work in our laboratory had shown that resistance was of unusually high level (parathion 84x, propoxur >1000x),[3] that it could not be significantly reduced by known synergists,[4] and was not due to enhanced metabolism of the insecticides.[5]

Although in most cases studied to-date resistance to OP and carbamate insecticides is dependent primarily on the biochemical alteration of the toxicant to non-toxic or less toxic products, in a small number of cases an apparently far more efficient mechanism of resistance has arisen consisting of insensitivity of the target enzyme, acetylcholinesterase (AChE), to inhibition by the toxicant. This property was known to exist only in acarines[6-8] but has recently been reported also in two species of insects, namely *Nephotettix cincticeps*[9] and *Musca domestica*.[10] Our results indicate that this is also the case with *Anopheles albimanus*.[3]

*Materials and Methods* - The parental strain (P) was collected in El Salvador in 1970 when it displayed low levels of tolerance to parathion, methyl parathion, and malathion. Part of P was subsequently selected in the larval stage by propoxur for 8 generations (Carb.-L strain), developing moderate to high resistance to carbamate, OP, DDT, and dieldrin insecticides.[4] A homozygous resistant strain (OP-R) was obtained by selecting Carb.-L with ethyl parathion for 17 generations. A second homozygous resistant strain (Carb.-R) was obtained by selection of P in the adult stage by propoxur, after outcrossing it to a 1972 collection from El Salvador. The susceptible strain (S) had been collected in Haiti in 1965, and was of normal susceptibility to OP, carbamate, and DDT insecticides.

The homogenates used for enzyme assays were made by grinding 1000 4th instar larvae in 0.1 *M* phosphate buffer at pH 7.5 in an ice-cold all glass homogenizer. The grinding ratio was 100 larvae per ml of buffer. The homogenate was centrifuged at 10,000 rpm for 1 hour and the supernatant used as the source of the enzyme.

AChE activity was determined by the method described by Ellman et al.[11] with acetylthiocholine iodide as the substrate. The incubation mixture consisted of 0.2 ml of enzyme pipetted into a 15 ml conical centrifuge tube which was maintained at 30°±1°C in a water bath. The enzyme was allowed to equilibrate for up to 2 minutes, then 50 μl of the inhibitor was forced under the surface of the preparation. The cholinesterase inhibition was stopped after different time intervals by diluting the mixture with 3 ml buffer solution containing $3.75 \times 10^{-3}$ M acetylthiocholine iodide and $2.0 \times 10^{-4}$ M DTNB. The amount of residual cholinesterase activity was measured at 412 mμ on a Unicam Sp-800 spectrophotometer. The reference cuvette contained 3 ml of the same buffer mixture that was used to quench the inhibition of the enzyme in order to cancel any spontaneous hydrolysis of the substrate.

The bimolecular rate constant ($k_i$) was measured for the following equation, in which E is the enzyme, I the inhibitor, and P the products.

$$\underbrace{E + I \rightleftharpoons EI \longrightarrow EI'}_{k_i} \longrightarrow P + E$$

The method of Aldridge[12] was used to calculate ($k_i$) values. The slopes of the plots were obtained by linear regression analysis.

Results and Discussion - The increases in resistance toward parathion, paraoxon, and propoxur in larvae of strain OP-R and Carb.-R as a result of their selection by parathion and propoxur, respectively, are indicated in Table 1. The resistance level of OP-R rose to 84x for parathion and 46x for paraoxon. The Carb.-R strain showed a 51.3x increase in cross resistance toward parathion. Both strains were almost immune to propoxur, their extrapolated $LC_{50}$ values having increased more than 2000x. However, the strains showed only a low level of tolerance toward fenthion, not exceeding 1.6x.

Table 1. COMPARATIVE LEVELS OF RESISTANCE IN *A. ALBIMANUS* SELECTED BY PARATHION (OP-R) AND PROPOXUR (CARB.-R)

| Compound | S $LC_{50}$ (ppm) | OP-R $LC_{50}$ (ppm) | Carb.-R $LC_{50}$ (ppm) | RR[a] | |
|---|---|---|---|---|---|
| | | | | OP-R/S | Carb.-R/S |
| Parathion | .008 | .67 | .41 | 84 | 51.3 |
| Paraoxon | .11 | 5.1 | - | 46 | - |
| Fenthion | .054 | .082 | .084 | 1.5 | 1.6 |
| Propoxur | .54 | 30%[b] | 36%[b] | >2000 | >2000 |

[a] Resistance ratio = $\frac{LC_{50} \text{ of R strain}}{LC_{50} \text{ of S strain}}$

[b] % kill with 1000 ppm propoxur

The values of the bimolecular rate constants ($k_i$) of the 4 strains are indicated in Table 2. Both OP-R and Carb.-R strains showed extremely high insensitivity of AChE to inhibition by propoxur amounting to 25,300x and 12,600x, respectively vis à vis the P strain. A pronounced decrease in sensitivity of AChE toward parathion in the OP-R and Carb.-R strains, amounting to 371x and 459x, respectively, is also evident.

Table 2. $K_i^a$ VALUES FOR LARVAE OF FOUR STRAINS OF A. ALBIMANUS

| Strains | Paraoxon | Propoxur | Fenoxon |
|---|---|---|---|
| Parathion-selected (OP-R) | $2.1 \times 10^3$ | $.19 \times 10^2$ | $1.5 \times 10^3$ |
| Propoxur-selected (Carb.-R) | $1.7 \times 10^{3b}$ | $.38 \times 10^2$ | |
| Parental (P) | $1.1 \times 10^6$ | $4.8 \times 10^5$ | |
| Susceptible (S) | $7.8 \times 10^5$ | $2.95 \times 10^5$ | $4.6 \times 10^3$ |
| Ratios: | | | |
| P/OP-R | 524 | $25.3 \times 10^3$ | |
| S/OP-R | 371 | $15.5 \times 10^3$ | 3.1 |
| P/Carb.-R | 647 | $12.6 \times 10^3$ | |
| S/Carb.-R | 459 | $7.8 \times 10^3$ | |

[a] Average of 4 replications, expressed as $\underline{M}^{-1}$ min$^{-1}$.
[b] Average of 3 replications.

An apparent anomaly in the relative sensitivity of AChE of the resistant strains was noted: a higher $k_i$ value (i.e. higher sensitivity) was found in the OP-R than in the Carb.-R strain when paraoxon was used as enzyme inhibitor, and the opposite occurred when propoxur was used as the inhibitor. However, the $k_i$ data are generally in agreement with the bioassay results showing high resistance to parathion and nearly complete immunity to propoxur in both strains.

As might have been expected, only a small difference in $k_i$ values was observed when fenoxon was used as the inhibitor since the levels of tolerance of the resistant strains toward fenthion are minimal.

The results in Table 3 indicate that the affinity of AChE of the R strain for acetylthiocholine, as represented by $k_m$ values, was 3.1x and 3.4x "poorer" than that of the S and P enzymes. These data are in agreement with results reported with house flies[10] and mites.[7] However, there was no significant difference in the affinity for acetylthiocholine among AChE of the Carb.-R, P, and S strains.

Table 3. MICHAELIS CONSTANTS (Km) AND MAXIMUM VELOCITY (Vmax) FOR HYDROLYSIS OF ACETYLTHIOCHOLINE BY LARVAL AChE OF FOUR STRAINS OF A. ALBIMANUS AT 30 ± 1°C, pH 7.5

| Strains | Km (M)[a] | Vmax (μ moles/L. per min)[a] |
|---|---|---|
| R | $16.7 \times 10^{-4\ a}$ | $13.3^{a}$ |
| C | $5.6 \times 10^{-4\ b}$ | $15.5^{b}$ |
| S | $5.3 \times 10^{-4\ b}$ | $25.4^{c}$ |
| P | $4.9 \times 10^{-4\ b}$ | $29.5^{d}$ |

[a] Any two means having the same superscript are not significantly different at 5% level as determined by Duncan's multiple range test.

The data for maximum velocity of hydrolysis of acetylthiocholine (Vmax) of the 4 strains showed significant differences. In general, Vmax values of the 2 R strains were almost half those of the P and S strains. These results are in agreement with similar data obtained on ticks[8] and mites,[6] and suggest that the AChE's in the 4 strains may be different isozymes.[13]

The data obtained suggest that the selection process resulted in the preponderance of individuals possessing an alteration in the active site of AChE which is involved in binding carbamate and OP insecticides. As a result, certain OP and carbamate compounds are no longer able to bind effectively to the altered site. Such alteration, however, does not confer insensitivity to all OP insecticides, as has been clearly demonstrated with fenoxon. It may be assumed that the S strains consist mostly of individuals with "sensitive" AChE. In the presence of individuals with "insensitive" AChE, the selective process is believed to proceed rapidly, since data obtained in our laboratory indicate that OP and carbamate resistance in this species manifests a high degree of dominance.

In view of the decisiveness of OP and carbamate resistance conferred on A. albimanus by AChE insensitivity, it is feared that such resistance may be a strong obstacle in the control of the species by these insecticides. However, the demonstration that insensitivity does not extend to fenoxon indicates that this property is at present specific for certain compounds or groups rather than generic.

## 2. Characteristics of Multiresistance in Culex quinquefasciatus

Introduction - Despite the development of high resistance to organophosphates in field populations of Aedes nigromaculis[14] and Culex tarsalis[15] in California during the past several years, the common house mosquito Culex quinquefasciatus has remained largely susceptible to these insecticides. However, reports were received of failures of chlorpyrifos to give adequate control of the species in the San Joaquin Valley in 1974. In view of the importance of C. quinquefasciatus as a vector of human disease worldwide, we initiated a study of the character of the emerging resistance and of the possibility of its suppression by synergists or alternative insecticides.[16]

Materials and Methods - Larvae in excess of 10,000 were collected in September, 1974, from dairy waste drains (Camara Dairy) approximately 4 miles from Hanford, California. These had been treated with various OP's for several years and with chlorpyrifos during 1973 and 1974. Additionally, it may be assumed that the mosquito population was indirectly exposed to chemicals applied to crops in the area.

The rearing and testing methods have been already described.[17] When synergism was tested, 0.5 ml of synergist was applied 4 hours in advance of the application of 0.5 ml of insecticide. When only insecticide was being tested, 1 ml volumes of the solutions were applied per 100 ml of water. All tests were performed on $F_1$ generation larvae.

Results and Discussion - Spectrum and Levels of Resistance - It is apparent from the data in Table 4 that the population of C. p. quinquefasciatus near Hanford has developed a wide spectrum of OP multiresistance. This comprises the main OP's used up to now in mosquito control in California, namely Abate®, at a level of 116.7x the normal $LC_{50}$, chlorpyrifos 52.2x, fenthion 48.9x, methyl parathion 24x, malathion 16.4x, and parathion 12.9x. Cross resistance obviously extends to related materials as indicated by a 12.4x increase in $LC_{50}$ for fenitrothion and 83.3x increase for chlorpyrifos-methyl. Neither of these chemicals has been employed for mosquito or agricultural pest control in the area.

The very limited cross resistance toward propoxur (2.7x) is noteworthy. Obviously the mechanisms of OP multiresistance in this strain do not significantly enhance carbamate metabolism. Absence of carbamate cross resistance was also observed in C. tarsalis from the Coachella Valley, whose multiresistance included chlorpyrifos-methyl 18.9x, fenthion 16.1x, chlorpyrifos 8x, fenitrothion 7.3x, methyl parathion 7.0x, Abate 3.9x, and malathion 3.3x.[18]

The resistance observed is believed to be of relatively recent origin, since the population had been found to be susceptible to OP's as recently as 1970 (G. P. Georghiou, unpublished). Furthermore, the

Table 4. RESISTANCE SPECTRA OF *CULEX P. QUINQUEFASCIATUS* AT HANFORD, CALIFORNIA, 1974

| Insecticide | Susceptible lab. strain | | Resistant field strain | | |
|---|---|---|---|---|---|
| | $LC_{50}$ (ppm) | slope (*b*) | $LC_{50}$ (ppm) | slope (*b*) | RR[a] |
| Abate | .0018 | 13.6 | .21 | 3.2 | 116.7 |
| Chlorpyrifos | .0023 | 7.0 | .12 | 6.1 | 52.2 |
| Chlorpyrifos methyl | .003 | 7.0 | .25 | 3.6 | 83.3 |
| Parathion | .0041 | 24.0 | .053 | 4.8 | 12.9 |
| Fenthion | .0047 | 10.8 | .23 | 6.1 | 48.9 |
| Methyl parathion | .005 | 19.1 | .12 | 4.0 | 24.0 |
| Fenitrothion | .017 | 10.1 | .21 | 4.9 | 12.4 |
| Malathion | .11 | 4.9 | 1.8 | 3.3 | 16.4 |
| Propoxur | .35 | 7.8 | .93 | 5.3 | 2.7 |
| Carbofuran | .052 | 3.8 | .14 | 4.6 | 2.7 |
| DDT | .065 | 5.6 | .39 | 1.3 | 6.2 |

[a] Resistance ratio = $LC_{50}$ resistant strain ÷ $LC_{50}$ susceptible strain.

levels of resistance at Camara are significantly higher than at a neighboring dairy 6 miles distant, suggesting that the populations have not as yet had adequate time to fully intermingle.

Results and Discussion - Synergism and Resistance - In order to gain an insight into the mechanisms of resistance a number of OP's were tested with the synergists piperonyl butoxide (p.b.), S,S,S-tributyl phosphorotrithioate (DEF), and triphenyl phosphate (TPP). The synergists were used at maximal sublethal concentrations of 5 ppm for p.b. and 2.5 ppm for DEF and TPP, irrespective of the dosage of insecticide tested. At higher concentrations DEF was approximately equitoxic to the two strains, the $LC_{50}$ levels being 29 ppm in the Camara and 31 ppm in the S strain, with similar slope values ($b$=2.4). Table 5 indicates the $LC_{50}$ values, synergism ratios and resistance ratios obtained. P.b. exhibited practically no synergism (or antagonism) of methyl parathion, methyl paraoxon, and chlorpyrifos in either the S or resistant (R) strain, the synergism ratios ranging from 1.0 to 1.3. In contrast, DEF exhibited a remarkable degree of synergism of OP's in the R strain, resulting in almost complete elimination of resistance. In all cases, DEF reduced the $LC_{50}$ values in the R strain to near the level of the unsynergized insecticides in the S strain. This effect was most pronounced in the case of chlorpyrifos.

Absence of synergism by p.b., a known inhibitor of the mixed function oxidase (MFO) system, would suggest that MFO detoxication enzymes are not involved in resistance in the strain studied. This would also explain, at least in part, the absence of carbamate resistance, since in a propoxur-selected strain of this species, MFO enzymes were shown to be primarily responsible for a higher rate of metabolism of propoxur, carbaryl, aldicarb, and carbofuran.[19,20]

Synergism of OP's by DEF and TPP has been reported earlier. Plapp et al.[21] observed strong synergism of malathion in *C. tarsalis* and suggested that the phenomenon was due to inhibition of the carboxyesterase detoxication enzyme. But subsequent observations of synergism of other OP's lacking carboxyester linkage[22,23] suggests that the action of DEF is broader, probably involving inhibition of a wider range of hydrolytic esterases. In this connection, Jao and Casida[24] have demonstrated that synergism of resmethrin and tetramethrin by DEF in various insects is due to inhibition of pyrethroid-hydrolyzing esterases. Hydrolytic enzymes were also found to be primarily responsible for metabolism of fenthion in *C. p. quinquefasciatus* from Burma, made 8-fold resistant by fenthion pressure in the laboratory.[25,26]

Results and Discussion - Alternative Chemicals - Table 6 presents the cross resistance spectrum of the Camara strain toward 12 new or experimental chemicals representing OP's, carbamates, organochlorines, pyrethroids, and insect growth regulators.

Table 5. SYNERGISM OF ORGANOPHOSPHATES BY PIPERONYL BUTOXIDE, DEF[a] AND TPP[b] AGAINST SUSCEPTIBLE AND OP MULTIRESISTANT[c] CULEX P. QUINQUEFASCIATUS LARVAE

| | S Strain | | | R Strain | | | Resistance ratio | |
|---|---|---|---|---|---|---|---|---|
| | Insecticide alone $LC_{50}$ (ppm) | Plus synergist $LC_{50}$ (ppm) | Synergism[d] ratio | Insecticide alone $LC_{50}$ (ppm) | Plus synergist $LC_{50}$ (ppm) | Synergism[d] ratio | Insecticide alone | Plus synergist |
| Synergist: Piperonyl Butoxide | | | | | | | | |
| Methyl parathion | .005 | .0048 | 1.0 | .12 | .12 | 1.0 | 24.0 | 25.0 |
| Methyl paraoxon | .025 | .024 | 1.0 | .4 | .31 | 1.3 | 16.0 | 12.9 |
| Chlorpyrifos | .0023 | .0020 | 1.2 | .12 | .094 | 1.3 | 52.2 | 47.0 |
| Synergist: DEF | | | | | | | | |
| Methyl parathion | .005 | .0012 | 4.2 | .12 | .0016 | 75.0 | 24.0 | 1.3 |
| Methyl paraoxon | .025 | .0057 | 4.4 | .4 | .007 | 57.1 | 16.0 | 1.2 |
| Parathion | .0041 | .0012 | 3.4 | .053 | .0028 | 18.9 | 12.9 | 2.3 |
| Chlorpyrifos | .0023 | .0024 | 1.0 | .12 | .0013 | 92.3 | 52.2 | 0.54 |
| Synergist: TPP | | | | | | | | |
| Malathion | .11 | .10 | 1.1 | 1.8 | .14 | 12.9 | 16.4 | 1.4 |

[a] S,S,S-tributyl phosphorotrithioate
[b] Triphenyl phosphate
[c] Camara strain, 1974
[d] Synergism ratio = $LC_{50}$ insecticide alone ÷ $LC_{50}$ insecticide in presence of synergist.

Table 6. RELATIVE TOXICITY OF NEW CHEMICALS AGAINST SUSCEPTIBLE AND OP-MULTIRESISTANT STRAINS OF *CULEX P. QUINQUEFASCIATUS*

| Insecticide | Susceptible strain | | Resistant strain | | |
|---|---|---|---|---|---|
| | LC (ppm) | Slope (*b*) | LC (ppm | Slope (*b*) | RR[a] |
| *Organophosphate* | | | | | |
| Chlorphoxim[b] | .0035 | 7.6 | .043 | 4.5 | 12.3 |
| Cyanox[c] | .017 | 11.1 | .29 | 3.9 | 17.1 |
| Celamerck S-2957[d] | .0013 | 10.9 | .048 | 3.9 | 37.7 |
| Ciba-Geigy 18809[e] | .042 | 7.9 | .15 | 6.3 | 3.6 |
| *Carbamate* | | | | | |
| CRC 11786[f] | .002 | 10.4 | .0037 | 3.8 | 1.9 |
| CRC 11783[g] | .013 | 6.4 | .018 | 4.7 | 1.4 |
| *Organochlorine* | | | | | |
| R. L. Metcalf XVI[h] | .054 | 6.1 | .039 | 4.9 | .7 |
| *Pyrethroid* | | | | | |
| Cismethrin[i] | .0016 | 5.3 | .0016 | 4.1 | 1.0 |
| Biopermethrin[j] | .0037 | 6.3 | .0030 | 5.0 | .81 |
| *Growth regulators* | | | | | |
| Methoprene[k] | .00029 | 2.4 | .0015 | 2.3 | 4.9 |
| Dimilin[l] | .0021 | 3.6 | .0031 | 3.5 | 1.5 |

[a] Resistance ratio, see footnote *a* Table 4

[b] *O*-chlorophenyl glyoxylonitril oxime *O*-ester with *O*,*O*-diethylphosphorothioate.

[c] *O*-*p*-cyanophenyl *O*,*O*-dimethyl phosphorothioate

[d] *O*-(2,5-dichloro-4-methylthiophenyl) *O*,*O*-diethylphosphorothioate

[e] *S*-(6-chloro-oxazolo(4,5b)pyridine-2(3H)-*O*-ylmethyl) *O*,*O*-dimethyl phosphorothioate.

[f] 2,2-dimethyl-2,3-dihydrobenzofuranyl-7 methyl(4-tert-butyl-2-methylphenylthio)carbamate.

[g] *O*-isopropoxyphenyl methyl(4-tert-butyl-2-methylphenylthio)carbamate.

[h] 2-(*p*-tolyl)-2-(*p*-ethoxyphenyl) 1,1,1-trichloroethane (Metcalf, et al., 1971).

[i] (5-benzyl-3-furyl)methyl (+)-*cis*-2,2-dimethyl-3-(2-methyl-1-propenyl)-cyclopropane-1-carboxylate

[j] 3-phenoxybenzyl *d*-*trans*-2,2-dimethyl-3-(2,2,-dichlorovinyl)cyclopropanecarboxylate.

[k] isopropyl 11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate

[l] 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)-urea

It is apparent that the broad spectrum of OP resistance observed (Table 4) extends also at relatively high levels to new experimental OP's such as Chlorphoxim, Cyanox, and Celamerck S-2957. Compound Ciba-Geigy 18809 is suspected to act as a carbamate after cleavage of the phosphate moiety, as described for *N*-phosphorothioylcarbamate esters by Fahmy et al.,[27] thus the relatively low resistance to it of 3.6x. Similarly, CRC 11786 and CRC 11783 are *N*-sulfenylated derivatives of the carbamates carbofuran and propoxur, respectively.[28] In the absence of carbamate resistance they are nearly as toxic to the R as to the S strain. Derivatization has resulted in a pronounced increase in larvicidal activity (approximately 26x over the parent compounds), thus placing them within the activity range of other common larvicides.

The OP-resistant strain is susceptible to RLM XVI, a biodegradable analogue of DDT,[29] despite its 5.7x resistance to DDT (Table 4). Of much interest is the absence of cross resistance to the synthetic pyrethroids cismethrin and biopermethrin. Unlike natural pyrethrins and related synthetic pyrethroids, biopermethrin is photostable[30] and thus holds promise as a larvicide against R strains.

Insect growth regulators are attracting much interest in mosquito control in view of their high activity, unique mode of action, and relatively low mammalian toxicity. Both methoprene and Dimilin manifest outstanding activity against both S and R strains. However, the cross resistance ratio of 4.9x toward methoprene should be noted, in view of the successful selection of *C. tarsalis*,[31] *C. p. pipiens*,[32] and *Musca domestica* (page 290, this report) for higher tolerance toward this compound.

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## O. JOINT ACTION OF HERBICIDES AND INSECTICIDES

### 1. Joint Action of Bipyridilium Herbicides with Propoxur and Fenthion

Introduction - While many investigators have shown the presence of interactions between herbicides and insecticides with respect to their toxicity to plants,[1,4] the effect of such combinations against insects does not appear to have been examined. Interactions in plants are most commonly synergistic due to inhibition of herbicide degradation in the presence of the insecticide[5-7] or due to inhibition of insecticide metabolism in the presence of the herbicide.[8]

The bipyridilium herbicides diquat and paraquat are broad spectrum herbicides which act by interfering with photosynthetic electron transport.[9,10] The herbicides undergo an oxygen-reversed one-electron reduction to form stable free radicals at fixed potentials (Eo).[11]

Phenazine methosulfate (Eo = +0.08v at pH 7.0),[12] which is well known to participate in biological redox reactions[13] has been shown to form a stable free radical on reduction[14] and is a potent inhibitor of microsomal oxidations (R. I. Krieger, unpublished). For a series of diquaternary compounds, inhibitory potency is dependent on the reduction potential (Eo) of the salts suggesting that inhibition is due to disruption of microsomal electron transport.

Diquat and paraquat find common usage in aquatic weed control.[9] At high concentrations (275 ppm), diquat enhances the in vivo toxicity to mosquito fish of carbaryl and Zectran® (4-dimethylamino-3,5-xylyl methylcarbamate) but does not affect the acute toxicity of aldrin, DDT, or parathion. This effect is associated with inhibition of the microsomal mixed function oxidase system of the animal and thus with impairment of its metabolic oxidative capability.[15]

Because the development of effective insecticide synergists is of great interest in studies of insecticide metabolism as well as in insect pest control, we investigated the possibility that quaternary compounds capable of interrupting microsomal electron transport could potentiate the toxicity of the insecticides propoxur and fenthion to mosquito larvae.[16] Insecticide-susceptible as well as resistant strains of mosquitoes were employed since it has been established that the higher insecticide tolerance of at least one of the resistant strains is largely due to enhanced mixed function oxidase activity.[17]

Materials and Methods - Diquat [6,7-dihydro(1,2-a:2',1'-c)pyrazinium dibromide (Compound III)] and its analogues [3,4-benzo-6,7-dihydrodipyrido-(1,2-a:2',1'-c)pyrazinium dibromide (II) and 7,8-dihydro-6H-dipyrido(1,2-a:2,1-c)-(1,4)diazepinium dibromide (V)] were synthesized by established methods.[11,18] Paraquat [1,1-dimethyl-4,4-bipyridylium dichloride (IV)] was obtained pure by crystallization of technical material. Phenazine methosulfate (I) was purchased from the Aldrich Chemical Co. and crystallized before use.

Most tests were performed on a strain of Culex pipiens fatigans (Wied.) of normal susceptibility to insecticides. Strains of C. p. fatigans resistant to propoxur (resistance level 30-fold) or fenthion (resistance level 30-fold) and a susceptible strain of Anopheles albimanus (Wied.) were also used but to a smaller extent. All bioassay tests were performed by established procedures.[19]

Results - Table 1 shows toxicity of the quaternary salts against insecticide-susceptible (S) strains of Culex and Anopheles. The compounds manifest extremely low toxicity to mosquito larvae, the concentrations required for kill far exceeding those being applied in practice for weed control, i.e., 0.5-5 ppm. The $LC_{50}$ values obtained contrast sharply with the corresponding values for common mosquito larvicides such as malathion, fenthion, and chlorpyrifos ($LC_{50}$ for C. p. fatigans 0.08, 0.0077, and 0.0022 ppm, respectively).[19] The propoxur-resistant (R) and fenthion-R strains of Culex were less susceptible to the herbicides by a factor of ca. 2-fold.

Tables 2 and 3 show the effect of pretreatment of larvae by the maximum sublethal dose of the quaternary compound, followed 2 hours later by application of propoxur. Against Culex, diquat at 200 ppm and paraquat and phenazine methosulfate at 100 ppm caused a definite increase in the toxicity of propoxur (Table 2). Diquat was the strongest synergist, producing a 2.2-fold increase in toxicity. The same compound was also strongly synergistic of propoxur against larvae of Anopheles (Table 3). Paraquat was much less synergistic against this species, and phenazine methosulfate showed no effect at a dose of 60 ppm. The degree of synergism demonstrated by the salts does not appear to be correlated with their relative toxicity to the larvae. However, synergism was dose dependent. Thus, sublethal concentrations of 100, 200, and 400 ppm diquat, when followed by application of 0.3 ppm propoxur, caused 75, 83.3, and 98.3% mortality in Culex larvae.

Diquat also was strongly synergistic with propoxur against the propoxur-R strain of Culex. Concentrations of 0.5, 1, and 1.5 ppm propoxur, producing <2.5% kill, resulted in 70, 77, and 100% kill when preceded by application of 1000 ppm diquat.

A limited number of tests with fenthion and diquat on fenthion-S and R strains of Culex also revealed a strong level of synergistic interaction by these compounds. Thus, against S larvae, fenthion at 0.006 ppm produced 23.8% mortality when used alone and 81.3% when preceded by 200 ppm diquat. Against R larvae, 0.05 ppm fenthion produced 1.2% alone and 86.2% when preceded by 400 ppm diquat.

Table 1. TOXICITY OF HERBICIDES TO 4TH INSTARS OF INSECTICIDE SUSCEPTIBLE STRAINS OF CULEX P. FATIGANS AND ANOPHELES ALBIMANUS

| Compound | Eo(v)[a] | Culex p. fatigans | | Anopheles albimanus | |
|---|---|---|---|---|---|
| | | $LC_{50}$ (ppm) | Max. sublethal (ppm) | $LC_{50}$ (ppm) | Max. sublethal (ppm) |
| Phenazine methosulfate (I) | +0.08 | 385 | 100 | 212 | 60 |
| Compound II | -0.18 | 1,000 | 200 | 705 | 80 |
| Diquat (III) | -0.35 | 690 | 200 | 475 | 100 |
| Paraquat (IV) | -0.45 | 275 | 50 | 1,000 | 100 |
| Compound V | -0.55 | 1,000 | 1,000 | 750 | 100 |

[a] Oxidation reduction potential (volts).

Table 2. JOINT ACTION OF PROPOXUR WITH SUBLETHAL CONCENTRATIONS OF HERBICIDES AGAINST 4TH INSTARS OF INSECTICIDE-SUSCEPTIBLE CULEX P. FATIGANS

| Propoxur (ppm) | % Mortality | | | | |
|---|---|---|---|---|---|
| | | Propoxur + Herbicide | | | |
| | Propoxur alone | + Compound II 200 ppm | + Diquat 200 ppm | + Paraquat 100 ppm | + Phenazine Methosulfate 100 ppm |
| 0 | 0 | 0 | 0 | 0 | 1.7 |
| .1 | 0 | 0 | - | - | - |
| .15 | 0 | 15 | - | - | - |
| .2 | 4 | 33 | 48.3 | 3.3 | 20 |
| .3 | 18.3 | 66 | 81.7 | 36.7 | 45 |
| .4 | 36.7 | 86 | 95 | - | - |
| .5 | 63.3 | 100 | - | 80 | 81.7 |
| .7 | 88.3 | - | - | - | 96.7 |
| $LC_{50}$ (ppm) | 0.44 | 0.25 | 0.2 | 0.35 | 0.31 |
| SR[a] | | 1.8 | 2.2 | 1.3 | 1.4 |

[a] SR (synergism ratio) = $\frac{LC_{50} \text{ propoxur}}{LC_{50} \text{ propoxur + herbicide}}$

Table 3. JOINT ACTION OF PROPOXUR WITH SUBLETHAL CONCENTRATIONS OF HERBICIDES AGAINST 4TH INSTARS OF INSECTICIDE-SUSCEPTIBLE *ANOPHELES ALBIMANUS*

| Propoxur (ppm) | % Mortality | | | |
|---|---|---|---|---|
| | | Propoxur + Herbicide | | |
| | Propoxur alone | + Diquat 100 ppm | + Paraquat 100 ppm | + Phenazine Methosulfate 60 ppm |
| 0 | 0 | 3.3 | 0 | 0 |
| 0.1 | 0 | 5.0 | - | - |
| 0.2 | 0 | 20 | 0 | 5 |
| 0.3 | 0 | 43.3 | 5 | 10 |
| 0.4 | 1.7 | 91.7 | 10 | 5 |
| 0.6 | 8.3 | 100 | 36.7 | 3.3 |

Discussion - The diquaternary salts and phenazine methosulfate exhibit only low to moderate toxicity to the mosquito larvae. The ionic nature of the salts could be expected to greatly restrict their absorption by the larvae and undoubtedly influences their low toxicity. In each case, the maximum sublethal concentrations are considerably below the established $LC_{50}$ values.

The detection of synergistic activity by these compounds, albeit at high concentrations, is significant in that it does support the hypothesis that compounds capable of blocking the microsomal electron flow in vitro can potentiate the toxicity of certain insecticides, possibly by inhibition of microsomal detoxication processes.

The compounds tested were selected to cover a range of redox potentials. Since diquat is the most effective synergist against both species of mosquito larvae, these results indicate that the mixed function oxidase activity of the larvae is susceptible to inhibition by electron transfer agents with redox potentials near that of diquat (Eo = -0.349v.).[11] This potential is well below that of other compounds such as quinones known to participate in microsomal electron transfer and suggests that other compounds more lipophilic than diquat with similar redox properties should be examined for synergistic activity.

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